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14. ABSTRACT Breast cancer is a highly diverse group of cancers and consists of at least 5 different subgroups. Furthermore, patients with Intra-tumor heterogeneity due to the presence of cancer cells with variable phenotypes such as different degrees of basal-like and luminal features increase the complexity of treatment. The aim of this project is to better understand the mechanisms that regulate breast cancer cell plasticity and origins of breast cancer heterogeneity. Our focus is the function of protein kinase D1 (PKD1), which is a serine/threonine kinase. We previously showed that PKD1 can repress epithelial to mesenchymal transition (EMT) by inhibitory phosphorylation of transcription factor Snail, a master switch of EMT. Supported by this award, we have performed experiments on molecular, cellular, mice xenografts and transgenic levels and conclude that *PKD1 is a context-dependent tumorigenesis and metastasis repressor or enhance.* Specifically, PKD1 is a metastasis repressor in luminal type breast cancer and loss of PKD1 in luminal type cells converts them into basal-like cells; and PKD1 is an enhancer of tumorigenesis in basal-like breast cancer. Based on current available data, conditional knockout PKD1 in mouse mammary tissue does not disrupt mammary development and does not induce tumorigenesis. However, when combination with P53 mutant, loss of PKD1 greatly enhances tumor incidence and metastasis rate, suggesting an anti-cancer role of PKD1—this study is still in progression, final results will be available after one more year.

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Introduction:

The aim of this project is to better understand the mechanisms that regulate breast cancer cell plasticity and origins of breast cancer heterogeneity. Breast cancer is a highly diverse group of cancers and consists of at least 5 different subgroups. Furthermore, patients with Intra-tumor heterogeneity due to the presence of cancer cells with variable phenotypes such as different degrees of basal-like and luminal features increase the complexity of treatment. The cellular origins of intra-tumor heterogeneity have been the subject of many recent studies. Both the cancer stem cell hypothesis and the clonal evolution model have been proposed to describe the establishment and maintenance of intra-tumor heterogeneity. In my own work, I have collected some evidence to support an alternative view, i.e. cancer cells have plasticity that manipulating certain signaling pathways can cause inter-conversion among different types of cancer cells. In other words, intra-tumor heterogeneity can arise from a subset cancer cells upon microenvironmental conditions and/or cell-intrinsic signaling. Our focus is the function of protein kinase D1 (PKD1), which is a serine/threonine kinase. Supported by this award, we have performed experiments on molecular, cellular, mice xenografts and transgenic levels. In this final report, I try to present data by standalone figures and tables with brief introduction and summary.

Body:

Aim (1): Protein kinase D1 (PKD1) is necessary for maintenance mammary luminal cell identity and loss of PKD1 results in reprogram luminal cell fate.

I. PKD1 IS A METASTASIS SUPPRESSOR GENE IN LUMINAL MCF7 CELL.

In our original application, we hypothesized that knockdown PKD1 in luminal MCF7 cells would induce epithelial to mesenchymal transition (EMT) and result in basal cell phenotypes. We have carried out multiple experiments at molecular and cellular levels to validate our hypothesis.

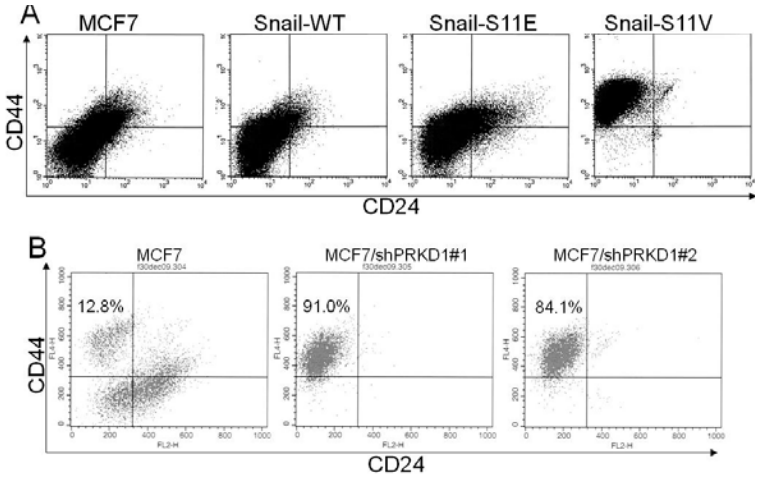


Fig 1. Flow cytometry analysis of breast cancer stem cell surface markers. *The $CD24^{lo}CD44^{hi}$ population contains so-called cancer stem cells [1]. (A) MCF7 stable cell lines that express wild-type (WT), S11E (mimic serine-11 phosphorylation), S11V (non-phosphorylation) mutant of Snai, a known EMT inducer [2, 3]. PKD1 can phosphorylate Snail at serine-11 and inhibit Snail transcription activity [4]. (B) MCF7 stable cell lines that express shPKD1 (from Open Biosystems. Two individual sequences were used). These data show that majority of MCF7 cells were transformed from luminal type cells ($CD24^{hi}CD44^{lo}$) into $CD24^{lo}CD44^{hi}$ basal-like cells when loss of PKD1 or expression of Snail S11V.*

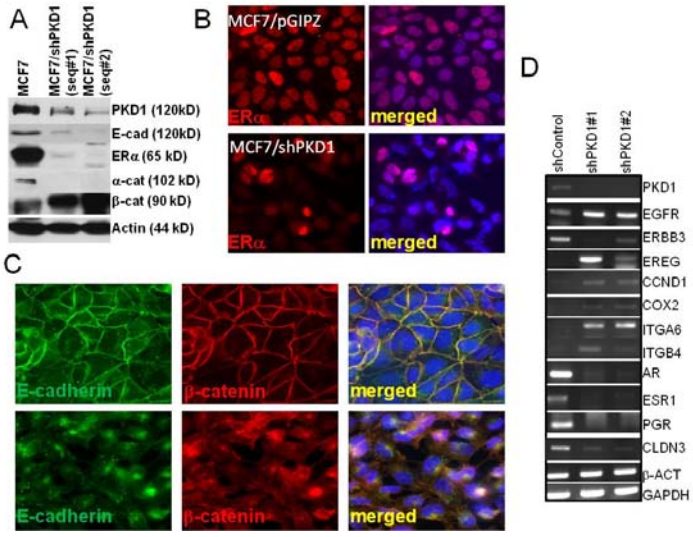


Fig 2. Loss of PKD1 induces EMT and down-regulation of ERα. *(A) Western blotting analysis indicates that knockdown PKD1 leads to down-regulation of expression of epithelial biomarkers, such as E-cadherin, α-catenin, as well as ERα. Expression of β-catenin is increased. (B and C) Immunofluorescence imaging to confirm down-regulation of ERα (B) and E-cadherin (C) in MCF7 cells expressing shPKD1. Note the dis-location of membrane E-cadherin and β-catenin in the MCF7/shPKD1 cells. Nucleus is in blue. Since multiple clones of MCF7/shPKD1 (no single clone selection) were used, ERα is still able to detect in some MCF7/shPKD1 cells. (D) RT-PCR analysis of gene expression in MCF7 and MCF7/shPKD1 cells. Gene symbol: ERα, estrogen receptor α; PgR,*

progesterone receptor; AR, androgen receptor; NR3C1, glucocorticoid receptor; EGFR, epidermal growth factor receptor, ERBB3, epidermal growth factor receptor 3;
EREG, epiregulin (a member of EGF family); ITGA6; Integrin alpha 6; ITGB4, Integrin beta 4; Cox2, Cyclooxygenase 2; IL 1B, Interleukin 1B; CCND1; cyclin D1; CLDN3, Claudin 3.

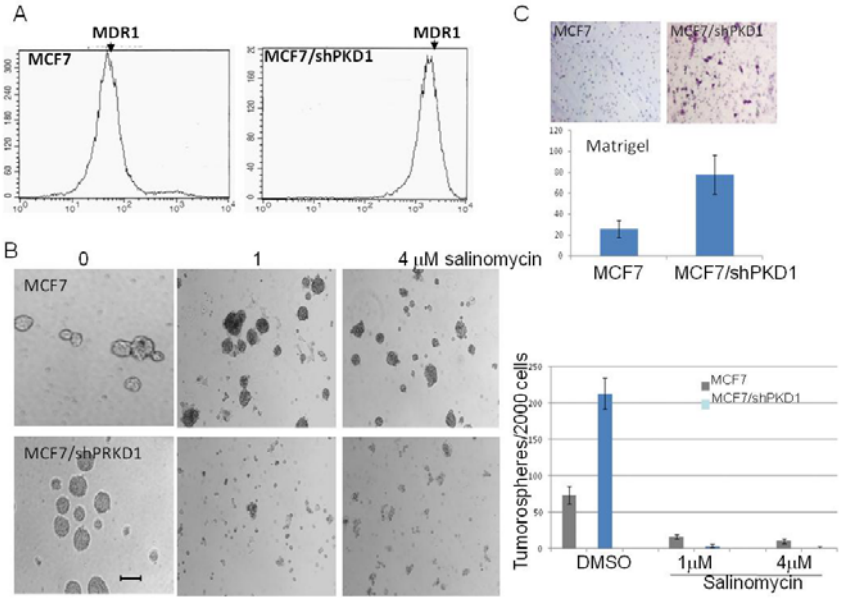
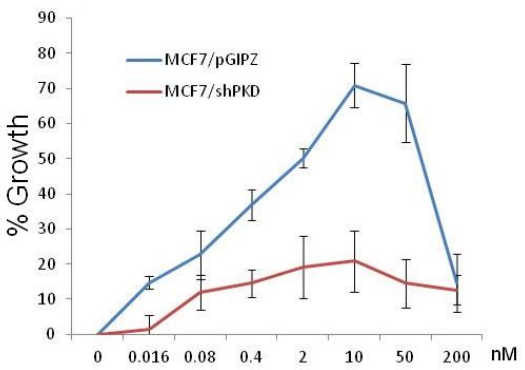


Fig 3. Inhibition of PKD1 generates CSC properties in MCF7 cells*. (A) Flow cytometry analysis of MDR1, a stem cell biomarker. Knockdown of PKD1greatly increases the expression of MDR1 in MCF7 cells. (B) Tumorsphere formation and salinomycin sensitivity. (left) Representative images of tumorspheres from MCF7 or MCF7/shPKD1 cells in the presence of salinomycin. The bar equals to 50 μm.* (right) *Quantification of tumorsphere formation. Bars denote the standard deviation for n=3. The MCF7/shPKD1 cells have about 3 times more tumorspheres than those from parental MCF7 cells.* (C) *MCF7/shPKD1 has higher invasion potential on matrigel assay.*

Table 1. Selected genes that up- or down-regulated by PKD1 in MCF7 cell*. Two individual shRNA targeting PKD1 were stably transfected into MCF7 cells. Genome-wide cDNA microarray (Affymetrix GeneChip Human Gene 1.0 ST Array) was performed to compare expression in MCF7 and MCF7/shPKD1. There are total 733 genes whose expression has a two-fold change. The genes in blue are down-regulated in MCF7/shPKD1. The genes in red are up-regulated in MCF7/shPKD1. The values are given as Log2 ratios. In general, the down-regulated genes include ERα and ERα-regulated genes, epithelial differentiation genes, pro-apoptosis and DNA repair genes. Many up-regulated genes are associated tumor aggressiveness and poor clinical outcome (PLAT, PLAU, CAV1/2, ITGA6 and ITGB4), and some are basal cell signature genes. Validation of the microarray data for some genes by RT-PCR is in Fig 2D.*

Gene symbol	Name	si1/MCF7	si2/MCF7 (log 2 ratio)	
PRKD1	PKD1	-3.24	-2.49	
ESR1	estrogen receptor 1	-13.69	-10.63	ER-regulated genes
PGR	progesterone receptor	-3.92	-3.79	
TFF1	trefoil factor 1	-12.63	-12.44	
IGFBP4	insulin-like growth factor binding protein 4	-4.4	-4.14	
PRLR	prolactin receptor	-5.27	-5.75	
BMP7	bone morphogenetic protein 7	-5.2	-4.96	
RERG	RAS-like, estrogen-regulated, growth inhibitor	-6.07	-6.61	
MUC1	mucin 1	-7.8	-7.84	
GATA3	GATA binding protein 3	-18.89	-14.9	epithelial differentiation
XBP1	X-box binding protein 1	-2.42	-1.77	
ELF5	E74-like factor 5 (ets domain transcription factor)	-10.33	-9.31	
SPDEF	SAM pointed domain containing ets transcription factor	-7.07	-6.14	
PDCD4	programmed cell death 4 (neoplastic transformation inhib	-4.99	-4.21	
BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	-20.72	-21.34	pro-apoptosis
TP53INP1	tumor protein p53 inducible nuclear protein 1	-3.24	-3.11	
AR	androgen receptor	-2.46	-2.46	
CTNNA1	catenin, alpha	-3.63	-4.29	
BRIP1	BRCA1 interacting protein C-terminal helicase 1	-3.02	-3.78	DNA repair
RAD51C	RAD51 homolog C (S. cerevisiae)	-2.3	-1.93	
CLDN3	Claudin 3	-2.22	-1.62	
PLAT	plasminogen activator, tissue	2.6	1.32	
PLAU	plasminogen activator, urokinase	2.6	1.84	
CAV1	caveolin 1	3.27	1.41	
CAV2	caveolin 2	2.46	1.5	
TM4SF1	transmembrane 4 L six family member 1	2.51	1.79	
EREG	epiregulin	23.6	11.3	
LAMB3	laminin, beta 3	2.69	2.25	basal cell signature genes associated aggressive tumor features
LGR5	leucine-rich repeat-containing G protein-coupled recepto	2.55	2.14	
TGFBR3	transforming growth factor, beta receptor III	2.08	1.89	
TGFB2	transforming growth factor, beta 2	1.52	2.07	
CTGF	connective tissue growth factor	1.93	2.47	
ETV4	ets variant gene 4 (ets-related molecule)	2.27	1.89	
ETV5	ets variant gene 5 (ets-related molecule)	2.37	1.72	
ITGA3	integrin, alpha 3	2.13	1.33	
ITGA6	integrin, alpha 6	2.27	1.54	
ITGB4	integrin, beta4	2.21	1.17	
BIRC3	baculoviral IAP repeat-containing 3	2.22	1.68	
KITLG	KIT ligand, stem cell factor	2.13	1.95	

Fig 4. MCF7/shPKD1 cells lost response to estrogen stimulation. *Hormone therapy is one of the most effective treatments for most ERα*



positive patients. However, triple negative breast cancer patients are insensitive to this therapy. The parental MCF7 cell is response to E2 stimulation. However, the MCF7/shPKD1 cell losses expression of ERα (Fig 2 and Table 1) and is not sensitive to E2 stimulation. MCF7 is an ERα positive cell line, however, knockdown PKD1 in MCF7 results in loss of ERa expression (Fig2 and Table 1). The result suggests that knockdown PKD1 can lead to resistance of tumor cells to endocrine therapy. METHOD: the cells were plated in 96-well at a density of 6000-8000 cells/well, in 1% dextran-coated-charcoal-pretreated FBS, phenol-red-free DMEM medium with indicated concentrations of E2 (17β-estradiol). The culture lasts 5 days with media change every 48 hr with fresh test compounds. Results from average of triplicate samples with standard errors. MCF7 cells are stimulated by E2 at physiological level (10 nM) and inhibited by high level of E2 (200 nM). The MCF7/shPKD1 cell is insensitive to E2.

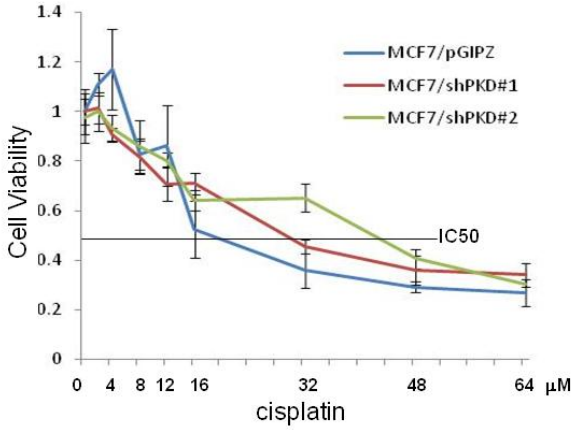


***Fig 5. Knockdown PKD1 makes MCF7 insensitive to cisplatin**. Dose-response curve of MCF7/pGIPZ (control) and MCF7/shPKD#1 and #2 after treatment of cisplatin at final concentrations 0, 2, 4, 6, 8, 12, 16, 32 and 64 μM for 3 days. The surviving fractions were determined by MTT assay. Each point represents the average percentage cell growth among four wells. Bars represent standard errors. The two MCF7/shPKD cell lines have higher IC50 values than that of control. The result suggests that knockdown PKD1 leads to acquired resistance of tumor cells to chemotherapeutic drugs.*

	# of cells	Inoculation	n mice	Lung Matastatic Colonization (2 month)	Tumor Incedence
MCF7/PGIPZ	1×10^6	IV	9	1	11.10%
	5×10^5	IV	9	none	0%
	5×10^4	IV	9	none	0%
MCF7/shPKD1	1×10^6	IV	9	3	33.30%
	5×10^5	IV	9	1	11.10%
	5×10^4	IV	9	1	11.10%

***Table 2. The MCF7/shPKD1 cells have more metastasis potential in mice xenografts**. Tail vein injection of MCF7/pGIPZ (control) or MCF7/shPKD1 cells into female athymic nude mice (6-8 weeks of age). Lung metastasis was monitored with MRI at 8 weeks after injection. All animal work was approved by UMASSMED IACUC protocol #2270. The subcutaneous xenograft experiment of MCF7/pGIPZ (control) and MCF7/shPKD1 is in progression.*

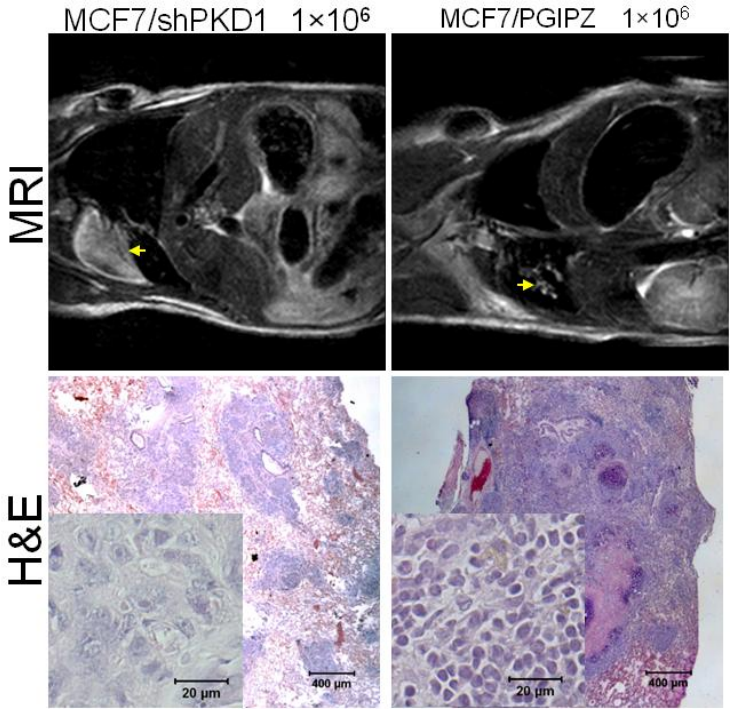


***Fig 6. Lung metastasis of MCF7/pGIPZ and MCF7/shPKD1**. Xenografted mice (tain vein injection) were examined for lung tumors by using MRI imaging (top panels). Yellow arrows indicate lung tumors. (bottom) H &E staining of lung tumors. Insets are enlarged images of tumors. More IHC staining with various antibodies is in progression.*

Summary: Our data strongly suggest that PKD1 is required to maintain epithelial identity of MCF7 cell. Knockdown PKD1 results in loss of expression of ERα and Progesterone Receptor and switches MCF7 cells into basal-like cells via epithelial to mesenchymal transition (EMT). The resulting MCF7/shPKD1 cells have basal cell properties, such as molecular signature and cellular behavior. More importantly, the resulting MCF7/shPKD1 cells have more potential to form lung tumors. Our results suggest that PKD1 is a metastasis suppressor gene in luminal MCF7 cell. Although the mechanisms of PKD1 action on suppression

EMT is still to be investigated, our previous studies suggest that transcription factor Snail is a potential downstream target for PKD1. As seen in Fig 1, the Snail S11V mutant, which mimics non-phosphorylated form, is the only isoform that can induce EMT in MCF7 cells and results in similar phenotype as knockdown PKD1.

II.PKD1 IS A POTENTIAL ONCOGENE IN BASAL-LIKE BREAST CARCINOMAS.

We initially hypothesized that ectopic expression of PKD1 in aggressive breast cancer basal cell line MDA-MB-231, a PKD1 negative cell line could lower its tumorigenesis and metastasis abilities. We carried out a serial molecular, cellular and animal experiments and conclude that ove-expression of PKD1 in MB231 cell promotes tumorigenesis and metastasis. Thus, we have modified our hypothesis that *PKD1 is a context-dependent tumorigenesis and metastasis repressor or enhance.*

Table 3. List of top genes that up- or down-regulated by overexpression of PKD1 in MB231 cells*.

	Fold of change	Symbol	Name
ups	38.53	NM_000689 // ALDH1A1 //	aldehyde dehydrogenase
	23.72	NM_000782 // CYP24A1 //	cytochrome P450, family
	21.88	NM_001354 // AKR1C2 //	aldo-keto reductase family
	12.13	NM_002742 // PRKD1 //	protein kinase D1 // 14q11 /
	11.57	NM_020299 // AKR1B10 //	aldo-keto reductase famil
	4.52	NM_000358 // TGFBI //	transforming growth factor,
	4.46	NM_002281 // KRT81 //	keratin 81 // 12q13 // 3887 /
	4.24	NM_004696 // SLC16A4 //	solute carrier family 16, m
	3.63	NM_000963 // PTGS2 //	prostaglandin-endoperoxide
	3.61	NM_000691 // ALDH3A1 //	aldehyde dehydrogenase
	2.69	NM_013230 // CD24 //	CD24 molecule // 6q21 // 10(
	2.65	NM_003739 // AKR1C3 //	aldo-keto reductase family
	2.59	NM_001432 // EREG //	epiregulin // 4q13.3 // 2069 /
	2.40	NM_001657 // AREG //	amphiregulin // 4q13-q21 //
	2.23	NM_181353 // ID1 //	inhibitor of DNA binding 1, dom
	2.16	NM_002166 // ID2 //	inhibitor of DNA binding 2, dom
	2.19	NM_021101 // CLDN1 //	claudin 1 // 3q28-q29 // 907
	2.19	NM_004360 // CDH1 //	cadherin 1, type 1, E-cadherin
downs	0.27	NM_000600 // IL6 //	interleukin 6 (interferon, beta 2
	0.32	NM_005450 // NOG //	noggin // 17q21-q22 // 9241 /
	0.33	NM_002640 // SERPINB8 //	serpin peptidase inhibito
	0.33	NM_005627 // SGK1 //	serum/glucocorticoid regulat
	0.37	NM_006795 // EHD1 //	EH-domain containing 1 // 11
	0.39	NM_000584 // IL8 //	interleukin 8 // 4q13-q21 // 357
	0.40	NM_001024465 // SOD2 //	superoxide dismutase 2,
	0.42	NM_175617 // MT1E //	metallothionein 1E // 16q13
	0.45	NM_005238 // ETS1 //	v-ets erythroblastosis virus E2
	0.47	BC133653 // MT1P3 //	metallothionein 1 pseudogene
	0.47	NM_138461 // TM4SF19 //	transmembrane 4 L six fa
	0.47	NM_006290 // TNFAIP3 //	tumor necrosis factor, alp
	0.49	NM_004464 // FGF5 //	fibroblast growth factor 5 // 4

** The expression profiling data was collected from microarray analyses using Affymetrix GeneChip (Human Gene 1.0 ST Array). Among genes with two-fold change in expression induced by overexpression of PKD1, there are 97 genes that are up-regulated and 316 genes that are down-regulated. On the up-regulated genes, PKD1 (green) is overexpressed by stable transfection. Genes highlighted by yellow color have been validated by RT-PCR in Fig 7. There are several types of gene of interest on the list, including stem cell specific markers (ALDH1A1 and ALDH3A1), epithelial markers (CD24, CDH1, CLDN1), EGF family members (AREG and EREG), steroid metabolism enzymes (AKR1C2, AKR1C3 and AKR1B10), vitamin D3 metabolism (Cyp24A1), inflammation modulator PTGS2/Cox2 and lactic acid transporter SLC16A4. Of the down-regulated genes, IL6 and IL8 are inflammatory cytokines that promote cancer progression and metastasis, SGK1 has a role to inhibit apoptosis of breast cancer cells. Since the genes listed mix of both known oncogenes and tumor suppressors, it does not give a clear clue to predict cellular behaviors.*

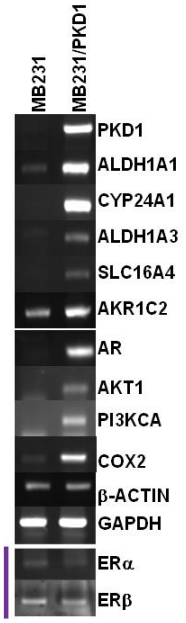


Fig 7. Validation of gene expression in MB231/GFP and MB231/PKD1. *RT-PCR was performed to confirm the up-regulated genes by overexpression include two cancer stem cell markers (ALDH1A1 and ALDH1A3). Other interesting top up-regulated genes include Cyp24A1, a vitamin D3 metabolism enzyme; SLC16A4, a lactic acid transporter; AKR1C2, a progesterone metabolism enzyme; AKT1 and PI3KCA are oncogenes to promote cell survival; Cox2 is involved in inflammation. The expressions of ERα and ERβ are not affected. Of particular interest, expression of androgen receptor (AR) is up-regulated by PKD1. Recently, a study suggested that the basal type breast cancer be further subtyped into five groups, one is luminal AR (LAR) group [5]. One characterization of the LAR group is expression of AR and response to anti-androgen therapy. We are testing if the MB231/PKD1 response to androgen stimulation. Nevertheless, the resulting MB231/PKD1 displays many cellular behaviors that are different from parental MB231 cell.*

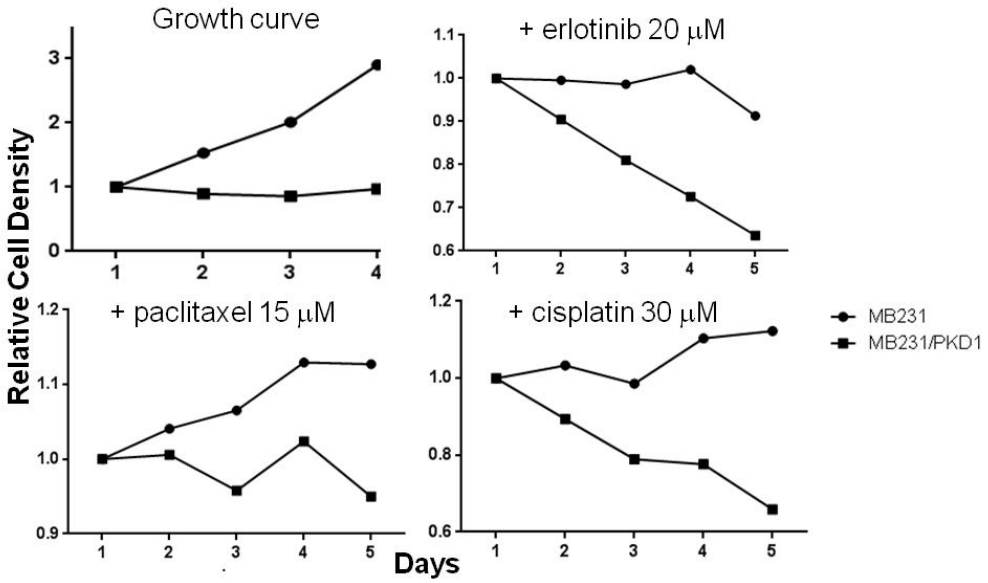


Fig 8. MB231 and MB231/PKD1 have differential sensitivity to therapeutic agents. *The triple negative breast cancer patients have few choices of therapeutic agents. The MB231 cell line is a basal type cell line and is resistant to EGFR inhibitor erlotinib, mitotic inhibitor paclitaxel and DNA damaging agent cisplatin. However, over-expression of PKD1 makes the cell line sensitive to erlotinib and cisplatin. Method: Cells were plated into 96-well plates at 2000-4000 cells/well. After overnight culture, indicated agents were added and continued for 5 days. Cell viability was measured by MTS method. Each point represents the average of three samples.*

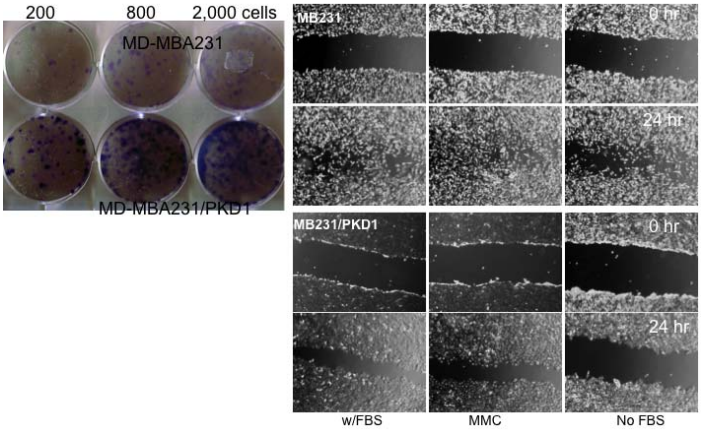


Fig 9. Clonogenic and migration assays*. (left) clonogenic assay. MB231 and MB231/PKD1 cells were plated in a 6-well plate at 200, 800 and 2000 cell/well and allowed to grow for 12 days. The cellular colonies were stained with Quike Diff (Fisher Scientific). The MB231/PKD1 cell has more colony formation ability. (right) Cell migration (wound healing) assay. MB231 and MB231/PKD1 cells were plated onto 6-well plates on confluent density. Cells were scratched off with a sterile pipet tip. To exclude possible contribution cell proliferation, either 3 μM of mitomycin C (MMC) was added into media or serum withdrawal to prevent cell proliferation. Images were taken 24 hours after scratching. The result indicates that MB231 has more migration ability.*

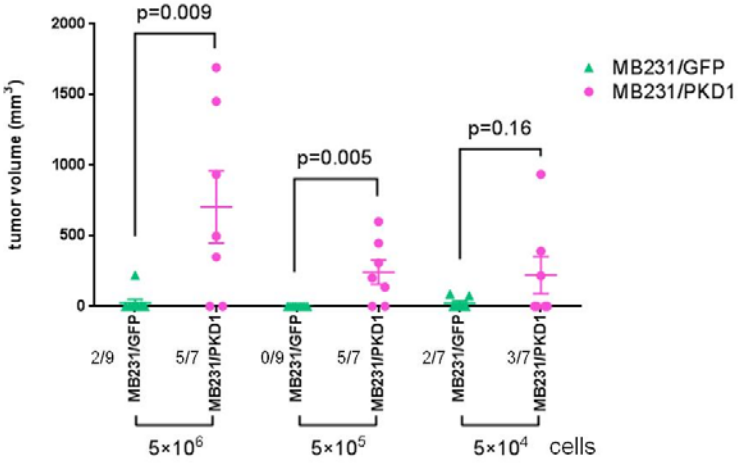


Fig 10. Tumor incidence and size for B231 and MB231/PKD1 in mouse subcutaneous xenografts*. The indicated numbers of cell were resuspended in 100 μl Matrigel diluted 1:2 in DMEM and injected in 6-8 weeks old, female athymic Nude mice (Taconic Farm) via subcutaneous route. Tumor sizes were measured at the 53rd day (9 weeks) after injection. Initial results indicate that MB231/PKD1 has more tumor formation potential than MB231. More data analysis and IHC study will be performed.*

Table 4. Table 2. The MB231/PKD1 cells have more metastasis potential in mice xenografts*. Tail vein injection of MB231 (control) or MB231/PKD1 cells into female athymic nude mice (6-8 weeks of age). Lung metastasis was monitored with MRI at 8 weeks after injection.*

	# of cells	Inoculation	n mice	Lung Matastatic Colonization (2 month)	Tumor Incedence	Number of foci / single mice
MB231/GFP	1×10^6	I.v.	9	2	22.20%	1 – 4
	1×10^5	I.v.	9	1	11.10%	2
	1×10^4	I.v.	9	none	0%	none
MB231/PKD1	1×10^6	I.v.	9	4	44.40%	2 – 12
	1×10^5	I.v.	9	2	22.20%	1 – 2
	1×10^4	I.v.	9	1	11.10%	1

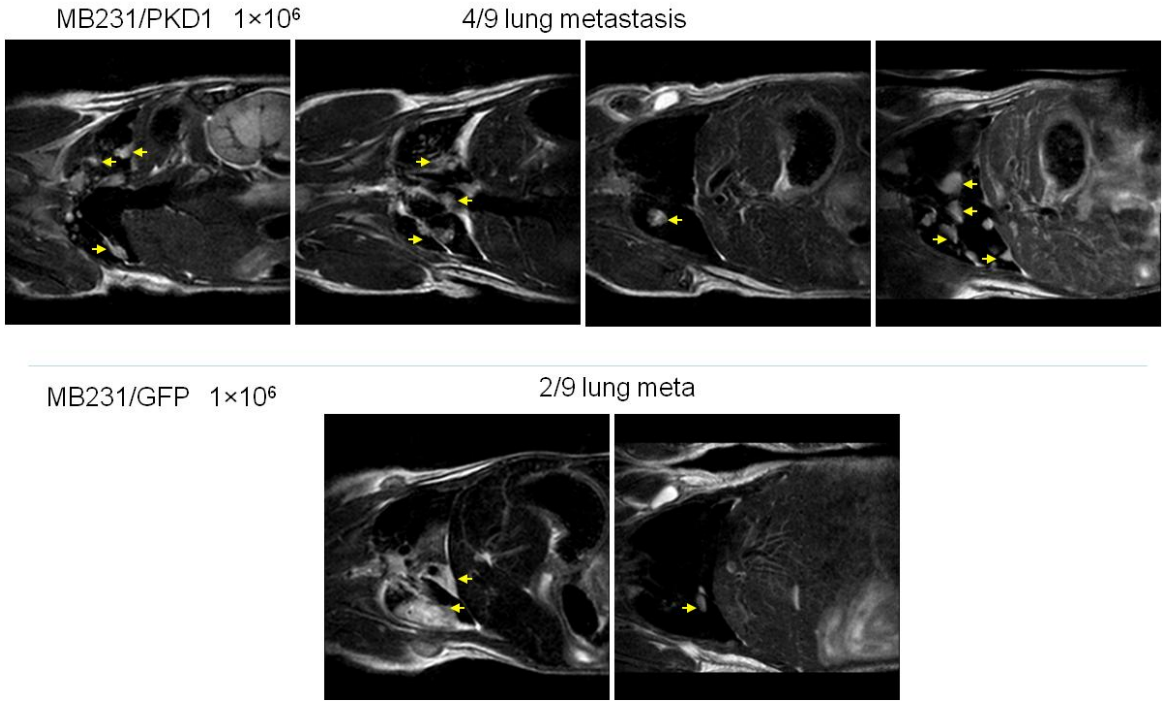


Fig. 11. Lung metastasis detected by MRI*. Xenografted mice (tain vein injection) were examined for lung tumors by using MRI imaging. Tumors were indicated by yellow arrows.*

Summary: Although the microarray and cellular behavior assays (clonogenic, migration and growth) data are somewhat confusing and contradictory, it is clear that the MB231/PKD1 cell has more potential in tumor formation and metastasis in mouse xenografts. These data suggest that PKD1 has a role in promoting tumor formation and metastasis in MB231 cell by a mechanism that reverse the MB231 cell back to stem cell like state with expression of AR. More molecular and cellular experiments are needed to confirm.

III. A POSSIBLE MOLECULAR MECHANISM FOR THE CELL-CONTEXT DEPENDENT PKD1 ACTIVITY.

To explain the dual role of PKD1 in MCF7 and MB231 cells, we think that one of our PKD1 studies provide some useful clues. In brief, PKD1 binds to E-cadherin in epithelial cell membrane and facilitate PKD1 activation. The kinetics of PKD1 activation is different in the presence or absence of E-cadherin. More importantly, PKD1 has different substrate phosphorylation profiles in the presence or absence of E-cadherin and it may suggest that PKD1 has distinct function in different cell types. A manuscript of the finding has been submitted to peer-reviewed journals for publication and is in Appendix 1.

Aim (2): PKD1 function in mouse mammary tissue development. We will generate mammary tissue specific PKD1 deletion mice and examine abnormality in mammary gland.

We have established genotyping PCR for Cre, PKD1 Lox/Lox and P53 mut/mut (Fig 12) and we are able to breed mice with expected genotypes.

(a) Generation of PKD1 knockout mice. We have bred male MMTV-Cre mouse (NCI mouse repository, #01XA9, line F) with female PKD1 Lox/Lox (kindly provided by Dr Eric N. Olsen, University of Texas Southwestern Medical Center). Further breeding of heterozygous Cre+; PKD1 Lox/+ mice leads to generate Cre+; PKD1 Lox/Lox mice, which have PKD1 deletion in Cre+ tissues including mammary glands.

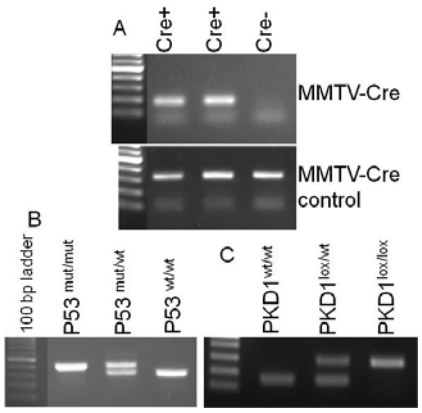
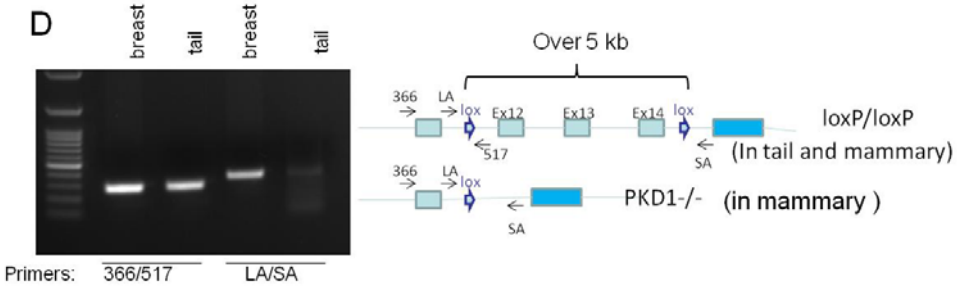
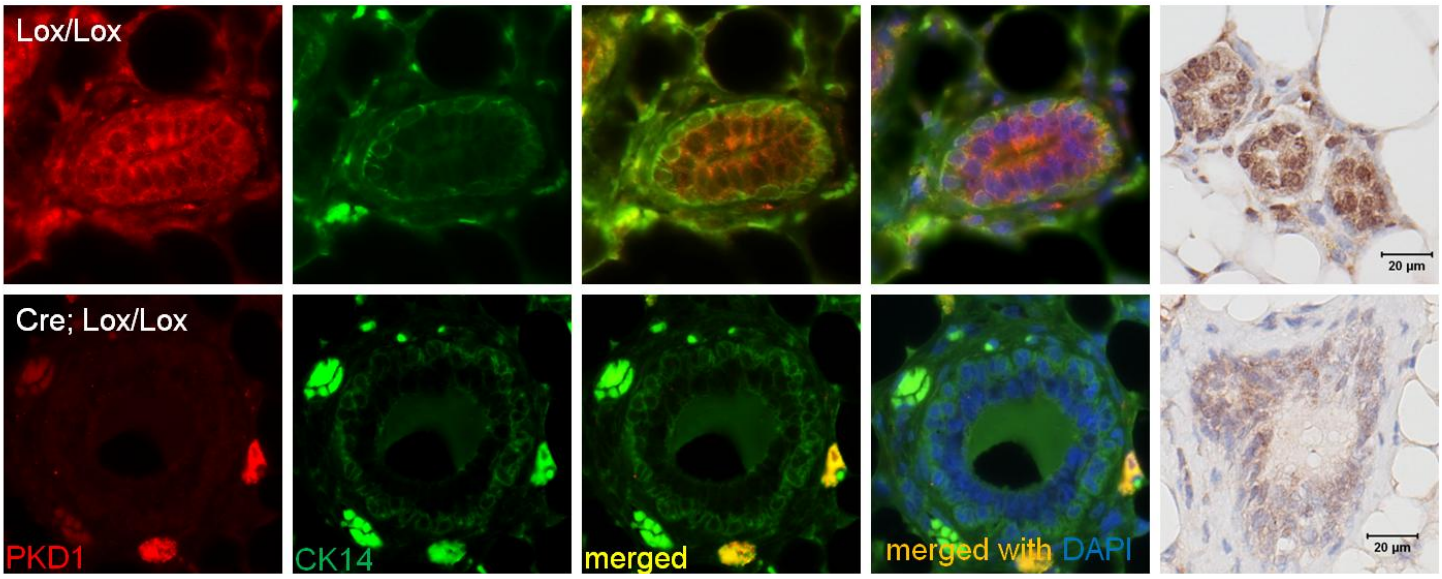


Fig 12. PCR based genotyping. *DNA extracted from mouse tail was used to perform genotyping to identify Cre (A), P53 mutant (B) and PKD1 Lox (C) alleles. In detecting P53 and PKD1 alleles, homozygous and heterozygous alleles are shown. (D) Mammary and tail DNAs were prepared for PCR. As shown on the left drawing, one set of PCR primers, LA/SA can amplify PKD1 deletion only in mammary tissue, but not in tail, suggesting that the Cre recombinase works in mammary tissue.*



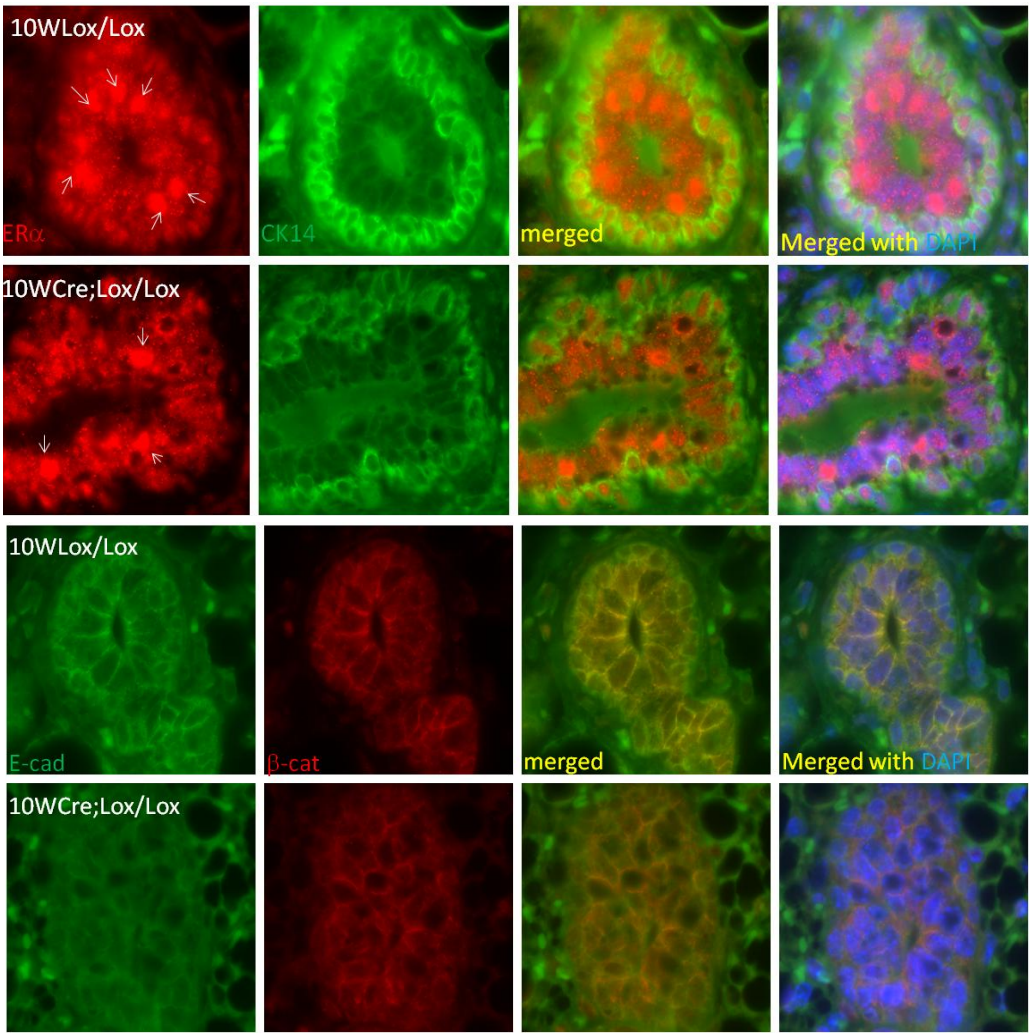
(b) Collection of mammary tissues at different stages and examination. After genotyping, female mice with desired genotypes were allowed to grow to certain age. We have collected PKD1 knockout mice with ages from 3 to 33 weeks. Currently, we have 17 PKD1 KO (Cre+; PKD1 Lox/Lox) mice, 11 littermate control (PKD1 Lox/Lox) and 4 Cre+ littermate mice. Two of the 17 PKD1 KO mice develop breast and lung cancer. Some preliminary immunostaining have been done.

Fig 13. PKD1 protein locates in luminal cells only in a mouse mammary . *Mouse mammary tissues were*



isolated and fixed in 10% Formalin/PBS solution and embedded into paraffin. For IF, anti PKD1antibody A20 (Santa Cruz Biotech) was used at 1:400 dilution. Basal cell biomarker cytokeratin 14 (CK14) antibody (clone LL02) was used at 1:400 dilution. For IHC, anti-PKD1 antibody (Sigma Chemicals, cat# K4892) was used at 1:200. The result suggests, for the first time in literature, that PKD1 protein expresses in luminal cells only. We have tried four (4) different PKD1 antibodies, the above two antibodies give the best results.

Fig 14. Expression of ERa and E-cadherin proteins are decreased in mammary gland of PKD1 KO mice. *ERa antibody (MC20, Santa Cruz Biotech) was used at 1:200 dilution for IF. The control mouse (10 weeks old PKD1 Lox/Lox) has more highly stained cells than those in PKD1 KO (white arrows). The E-cadherin antibody (E36, from BD) and b-catenin antibody (H108, Santa Cruz) were used at 1:200 dilution. Quantitative measurement and statistical analysis method are needed to develop.*



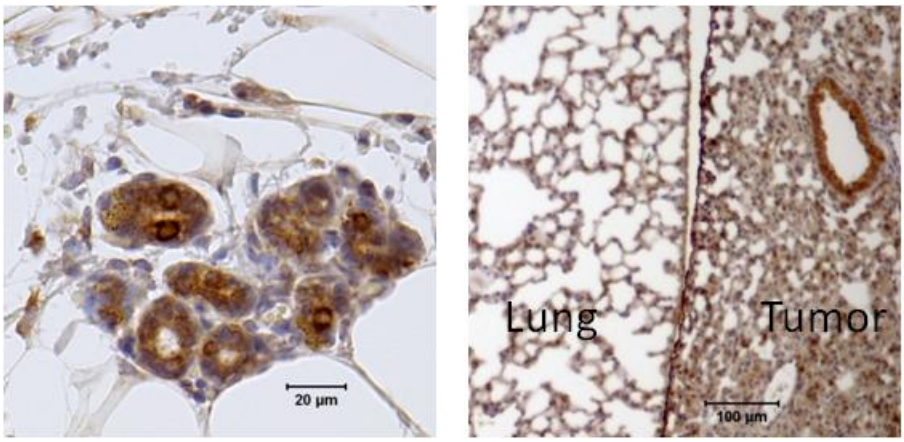


Fig 15. PKD1 KO mice develop breast and lung tumors. *A 26 weeks old PKD1 KO mouse develops breast (left) and lung tumor (right). Staining with CK8 antibody. We will perform more CK8 staining for luminal cells and CK14 staining for basal cells to fulfill Task (c) in Aim 2 of SOW, which states "Mammary epithelial cell compositions in PKD1 knockout mice".*

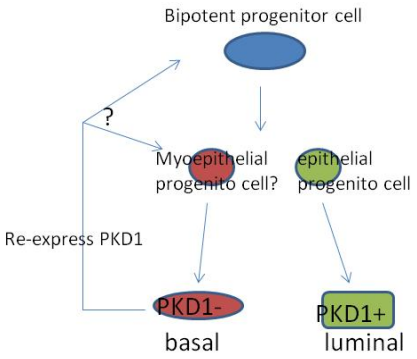
KEY RESEARCH ACCOMPLISHMENTS:

- Propose an alternative hypothesis, other than stem cell or clonal evolution to explain the intra-tumor heterogeneity. Supported by this grant, we have collected experimental data to support our hypothesis.
- Illustrate Protein kinase D1 role in breast cancers and may help designing personal therapeutic regimen for PKD1 positive and negative breast cancer.
- To our best knowledge, we are the first to carry out PKD1 knockout experiment in mouse mammary tissue.

REPORTABLE OUTCOMES:

- One manuscript has been submitted for peer-reviewed journal (Appendix 1), one is in writing.
- One postdoctoral researcher (Dr Zhuo Li) has been trained and now appointed as an oncological surgeon in a China hospital.
- Multiple cell lines (two for MCF7/shPKD1, one for MDA-MB231/PKD1, one for SKBR3/PKD1). Multiple mouse knockout PKD1 mammary tissues and parallel control have been collected.
- I myself is preparing next grant application based on the results from this award project.

CONCLUSION: We have conducted comprehensive studies at molecular, cellular and animal model levels to illustrate our hypothesis that luminal type of breast cancer cell can be converted into basal-like cell by manipulating PKD1 signaling. In normal mouse mammary development, PKD1 expression is limited in luminal cell only. PKD1 functions in maintenance of epithelial cell identity. In luminal cancer cell line MCF7, loss of PKD1 results in downregulation of E-cadherin, ERα and ERα-regulated genes. Knockdown PKD1 in MMTV-Cre mice, the luminal cells (CK8 positive) express less ERα and E-cadherin. More importantly, we have observed that 2/17 PKD1 KO mice develop breast and lung tumors, suggesting PKD1 plays a critical anti-cancer role in mammary gland. According to currently available data, we propose a mode of PKD1 action (left). The function of PKD1 in breast cancers is dual, in luminal type cancer, it has a cancer suppressor role; in basal-like cancer, it promotes tumor. Effects for further analysis of normal/cancer stem cells are hampered by lack of single distinct stem cell markers with anatomical position information, such as LGR5 in small intestine stem cell [6]. We have noted that many triple negative cancers and many basal-like cancer cell lines express PKD1, it is interesting to know how PKD1 contribute to tumor progression and metastasis. For future work on PKD1, I suggest: (1) test other promoter driven Cre (such as WAP-cre) to knockout PKD1 and study PKD1 function during pregnancy and lactation; (2) knockin PKD1 into mammary basal cells using CK14-Cre mouse with P53 mutant background. This mouse model may mimic some human triple breast cancer with P53 mutant and expressing PKD1 and this approach will help to understand how PKD1 acts in basal-type cancer cells; (3) using existing small molecule inhibitors for PKD1 to treat breast cancer in mouse models. As we suggest that expression of PKD1 in basal-like cancer cells promotes tumor progression, it is possible to inhibit PKD1 will suppress tumor; and (4) data mining to surrogate correlation of PKD1 expression with clinical outcomes of breast cancer patients with different genetic background and subtype of tumors.



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Appendix 1

E-cadherin facilitates protein kinase D1 (PKD1) activation and subcellular localization

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Running title: *PKD1 activation requires E-cadherin*

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Keywords: PKD1; activation; E-cadherin; membrane recruitment; subcellular localization

Background: PKD1 has diversified functions and substrates in many cells, but mechanisms of regulation PKD1 remain uncertain.
Results: PKD1 binds to E-cadherin as membrane anchorage and results in distinct kinetics, localization and substrate phosphorylation.
Conclusion: E-cadherin is important for spatial and temporal organization of PKD1 signaling.
Significance: Protein-protein interaction contributes to specificity of PKD1 signaling in cell type specific context.

SUMMARY
The current model for Protein kinase D1 (PKD1) activation involves diacylglycerol binding to the C1 domain of PKD1 and translocation to membrane where PKD1 is phosphorylated by protein kinase C (PKC) at the activation loop. In this study, we show that the intracellular domain of membrane protein E-cadherin binds to PKD1 via second cystein-rich (C1b) and catalytic domains. The binding leads to subcellular redistribution of PKD1: expression of full-length E-cadherin tithes more PKD1 to plasma membrane; while expression of cancer-related soluble cytoplasm fragment CTF2 results in accumulation of PKD1 in cytosol. This binding event has functional significance, as PKD1 has dramatically different kinetics during activation in the presence or absence of E-cadherin. Furthermore, artificial targeting PKD1 to plasma membrane leads to PKD1 activation in a PKC-independent manner, indicating that membrane attachment is sufficient to activate PKD1. Remarkably, PKD1 has different substrate phosphorylation profiles in the presence and absence of E-cadherin. Our results suggest that E-cadherin serves as a membrane attachment site for PKD1 in epithelial cells. We propose that protein-protein interactions facilitate spatial and temporal organization of PKD1 signaling cascade to ensure the speed and precision of substrate phosphorylation.

INTRODUCTION

When a cell responds to a stimulus, signaling pathways serve as on-off switches to transmit the message in. The molecular mechanisms that control the specificity of signaling have been studied extensively. Protein kinase C family has provided a good example [reviewed in Ref (1,2)]. PKC isoforms serve diverse (and in some cases opposing) functions in cells, however, they share general mechanisms in activation and substrate phosphorylation. Classic PKC members can be activated by diacylglycerol (DAG) and calcium signals. How can PKC multiple isoforms in the cell react differently to same second messenger signal input? It is at least in part as a result of protein-protein interaction leading unique subcellular localization. Several classes of PKC interacting proteins have been identified, including Receptors for activated C kinases (RACKs), Substrate-binding proteins (SBPs) and Proteins that interact with C kinase (PICKs) (3). These adaptor proteins have in common the ability to bind specific PKC isozymes and connect them to a particular organelle or site in the cell (4).

Protein kinase D1 (PKD1), the founding member of PKD kinase family, is crucial for development since PKD1 knockout is embryonic lethal in mice (5) and the PKD1 function is not complemented by two close family members, PKD2 and PKD3. PKD family is classified within the calcium/calmodulin kinase (CAMK) superfamily, however, PKDs share many structural and regulatory similarities to the PKC family and previously identified as PKC family members (6,7). The PKD family members share certain basic structural features, including cysteine-rich region (C1 region, subdivided into C1a and C1b regions), a pleckstrin homology (PH) domain and catalytic domain (6,7). Like PKC, PKDs show no or low catalytic activity in unstimulated cells through an autoinhibition mediated by the PH domain (8). Previous studies have demonstrated a general model of activation of PKD1 (7). In brief, PKCs and

PKD1 are recruited to the membrane surface in response to second messenger DAG. Activation of PKD is dependent on PKC activity to phosphorylate two key serine residues at PKD1 activation loop (Ser-744 and -748 in mouse PKD1). Activated PKD1 can autophosphorylate Ser-916 residue, an event which is often used as a surrogate marker for PKD1 activity. More recent studies show that PKD1 activation is more complicated. For example, G protein-coupled receptors induce not only a rapid PKC-dependent activation phase involving Ser-744 and -748 phosphorylation, but also a prolonged PKC-independent activation phase with Ser-748 autophosphorylation (9,10). Furthermore, dextran sulfate, a potent PKD1 agonist, increases high level S916 phosphorylation, but low/none Ser-744/748 phosphorylation (11).

Although phosphorylation plays a major role in PKD1 signaling efficiency or specificity, docking interactions with protein scaffolds or protein substrates also may contribute to the allosteric control of PKD1 activity (12). The A-kinase anchoring protein AKAP-Lbc assembles an activation complex including PKD1, PKCeta and PKA in cardiomyocytes. In this protein complex, PKD1 is activated by nearby localized PKC and release from the complex mediated by PKA activity (13). PKD1 was previously reported to co-localize and co-purify with E-cadherin, a major protein component in cell-cell adherens junctions (14). In this study, we show E-cadherin facilitates PKD1 plasma membrane translocation and activation.

MATERIALS AND METHODS

Plasmid constructs: The GFP-PKD1 was described previously (14). The P281G-PKD1 [equivalent to mouse PKD1 P287 (9,15)] which contains a point mutation in proline-281 into glycine residue in the C1b domain was generated by PCR-based mutation with Quikchange kit (Stratagene). The primer used (forward) 5'-GTCATCCACTCCTACACCCGGGCCACA GTGTGCCAGTACTGCAAG. The NLS-PKD1 was generated by inserting SV40 nuclear localization signal (NLS, with amino acid sequence PKKKRKV) downstream of GFP. Mem-PKD1 was created by inserting the sequence encoding the first 20 amino acid residues of neuromodulin upstream of GFP-PKD1 (Fig 1A). Detail methods and primer sequences are available on request. E-cadherin CTF2-pcDNA3 construct (Ref (17), Fig 3A) was kindly provided by Dr Y. Fujita, University College London. This construct contains the whole intracellular domain. IL2R-EcadC (Fig 3A) is a chimera that fuses the extracellular and transmembrane domains of the interleukin-2 receptor α subunit to the cytoplasmic domain of E-cadherin (Ref (18), kindly provided by Dr. C. Gottardi, Northwestern University). For yeast two-hybrid assay, the pGBK-T7 plasmid (Clontech) containing C1a, C1b, PH and catalytic domains of PKD1 were described previously (19). E-cadC-pGAD-T7 vector contains part of trans-membrane and whole intracellular domain of E-cadherin (amino acid residues 716-883). A full-length E-cadherin expression vector (Fig 3A) was obtained from Addgene.org (Plasmid #18804). ShRNAs targeting E-cadherin were purchased from Open Biosystems/Thermo.

Cell culture and immunofluorescence staining. Prostate cancer cell line LNCaP (from ATCC) was cultured in RPMI medium plus 10% unheated FBS. Prostate cancer cell line C4-2 was purchased from Uroco, Inc and cultured in DMEM plus 10% FBS. The stable C4-2 cell line that expresses PKD1-GFP (C4-2/PKD1) was described before (14). A C4-2 cell line that stably expresses full-length E-cadherin (C4-2/Ecad) was established by antibiotic selection of transit transfected C4-2 cells with the E-cadherin expression vector. The Colon cancer cell line SW480 (from ATCC) was cultured in L15 medium plus 10% FBS. Bryostatin 1 (from Sigma) was added to culture media to a final concentration of 10 nM. In Fig 2C, general PKC inhibitor GF 109203X (GFI, from Sigma) was added to cell culture 1 hour before adding Bryostatin 1 to a final concentration of 1 M. For immunofluorescence imaging, cells cultured on glass discs were fixed in 5% Formalin in PBS for 10 minutes at room temperature.

After washing, primary antibody was diluted in PBS solution with 0.3% Triton X-100 and 10 mg/ml BSA and incubated with fixed cells from 1 hour to overnight following by incubation with secondary antibody conjugated with appropriate dyes (all from Jackson ImmunoResearch). Images were taken by an Olympus IX-51 microscope equipped with SPOT software. Transient transfection was performed with Lipofectamine 2000 (Invitrogen).

Electrophoresis and Western blotting. Cultured cells were lysed in RIPA buffer (20) containing protease and phosphatase inhibitor cocktails (Sigma Chemicals) and centrifuged to removed insoluble debris. The protein samples were separated on 10% SDS-PAGE and transferred to PVDF membranes using a semi-dry transfer devise (Thermo Scientific) at constant current model (0.2 A). The membrane was then incubated with TBST (50 mM Tris-HCl, pH7.4, 100 mM NaCl and 0.2% Tween-20) plus 3% nonfat dry milk to block non-specific binding for 1 hour at room temperature. Incubation with primary antibodies was performed at 4 C for overnight. An E-cadherin antibody recognizes the intracellular domain (clone C36) was purchased from BD Transduction Laboratories and an antibody that recognizes the extracellular domain was from Santa Cruz Biotechnology. PKD1 and phosphorylated serine-916 (pS916) PKD1 antibodies were purchased from Epitomics. β-catenin, β-actin and GFP antibodies were purchased from Santa Cruz. The development and characterization of β-catenin phosphorylated Threonine-120 antibody was previously described (21). A phospho-(Ser/Thr) PKD1 substrate antibody which preferably detects LXR(Q/K/E/M)(M/L/K/E/Q/A)S*XXXX ((22)) was purchased from Cell Signaling. The densitometric quantification of immunoblotting was carried out with Image J software.

RESULTS

E-cadherin physically interacts with PKD1 and knockdown E-cadherin decreases PKD1 activity. PKD1 was previously reported to co-localize with E-cadherin (14). Since PKD1 was also reported to bind directly to β-catenin which is known in a protein complex with E-cadherin (23), it is uncertain if PKD1 association with E-cadherin is through a direct or indirect binding. To explore direct interaction, we tested on yeast 2-hybrid assay using individual PKD1 domains, C1a, C1b, PH and catalytic domain for direct interaction with E-cadherin intracellular domain. As seen in Fig. 1A, the C1b and catalytic domain of PKD1 can directly bind to E-cadherin intracellular domain. The C1b domain of PKD1 is required for binding to DAG and plasma membrane attachment during activation (7,24). PKD1 and E-cadherin can be co-immunoprecipitated reciprocally from C4-2/PKD1 cell lysate (Fig 1B), suggesting that the two proteins are within the same complex in mammalian cells. Since PKD1 is a protein kinase, we also tested in vitro phosphorylation of E-cadherin by PKD1. PKD1 was not able to phosphorylate purified E-cadherin cytoplasm fragment (data not shown). However, we found that expression a shRNA vector that targets E-cadherin in LNCaP, an E-cadherin positive cell line, can reduce PKD1 activation as judged by S916 autophosphorylation (mouse PKD1 animo acid residue, equivalent human PKD1 residue S910) and β-catenin T120 trans-phosphorylation (Fig 1C). When GFP tagged PKD1 and a shRNA targeting E-cadherin were co-transfected into LNCaP cells, PKD1 predominantly localizes in cytosol of resting cells and knockdown of E-cadherin does not result in noticeably changes on PKD1 localization. Stimulating agents, such as phorbol esters or Bryostatin 1, a potent PKD1/PKC activator (25) and possible anti-cancer drug candidate (26), induce PKD1 translocation to different cellular membranes, particularly plasma membrane in cells with control shRNA. In contrast, more PKD1 retains in cytosol in the absence of E-cadherin (Fig 1D).

Artificial targeting PKD1 to membrane activates PKD1 in the absence of simulations. To further study how binding to E-cadherin

might be required for PKD1 activation in vivo, we tested whether membrane recruitment of PKD1 might play a role in activation by artificially targeting it to the membrane. We made a fusion protein construct consisting of the first N-terminal 20 amino acid residues of neuromodulin and full-length PKD1 (mem-PKD1, Fig 2A). This neuromodulin fragment contains a palmitoylation signal that targets fusion proteins to membranes (27). When transfected into SW480 cell line, majority of this fusion protein localizes to plasma membrane and small fraction of the protein attaches to organelle membranes (Fig 2E), while wild type PKD1 localizes in cytoplasm (Fig 2D). Immunoblotting for pS916 shows that the mem-PKD1 is activated in the absence of stimulation. In contrast, the endogenous PKD1 remains inactive (lane 5, Fig. 2B) until Bryostatin was added (lane 6).

Both C1a and C1b domains of PKD1 can bind to DAG, however, C1b domain is responsible for the majority of DAG binding (15). Mutation of mouse PKD1 proline-287 residue (equivalent human PKD1 residue P281), a critical DAG binding site within C1b domain abolishes DAG/phorbol ester binding, decreases PKD translocation from cytosol to plasma membrane and prevents PKD activation (9,15,28). Indeed, the P281G mutant is not able to be activated by Bryostatin as indicated by phosphorylation status of S916 (Lane 4, Fig 2B). DAG has three functions in activating PKD1: (1) promoting PKD1 membrane translocation; (2) anchoring PKD1 on membrane via hydrophobic tails of DAG; and (3) activating PKCs as upstream kinase activator. To distinguish the three functions of DAG, we examine the activation of mem-PKD1 with P281G mutation (P281G/mem-PKD1). Like mem-PKD1, the P281G/mem-PKD1, but not the endogenous PKD1, becomes activated without stimulation (lane 7), suggesting targeting wild type or P281G mutant PKD1 to membrane mimics the DAG-induced membrane translocation, activating response normally induced by DAG.

Previous studies suggest that PKCs are the upstream kinases to activate PKD1 in canonical pathway [see review in Ref (7)]. Since the mem-PKD1 can be autophosphorylated in resting cells without upstream active PKCs, we wonder if the activation of mem-PKD1 in resting cells is a PKC-independent event. Indeed, incubation with PKC inhibitor GFI prevents activation of endogenous PKD1 (lane 6, Fig 2C), but does not block activation of the mem-PKD1 in the absence or presence of Bryostatin 1 (Lanes 5 and 6, Fig 2C), suggesting that the auto-activation of mem-PKD1 is PKC-independent. Although the exact upstream kinase(s) is elusive, it is certain that targeting PKD1 to plasma membrane brings PKD1 close to the un-identified activator(s).

E-cadherin binding alters PKD1 subcellular localization. When transfected into SW480 which is E-cadherin negative, the wild type GFP-PKD is localized in cytoplasm (Fig 3B). Co-transfected with full-length E-cadherin, the two proteins are co-localized on cytoplasm and membrane (Fig 3C), suggesting that the two proteins form a complex in vivo and expression of E-cadherin can affect PKD1 subcellular localization. To further examine how PKD1 subcellular localization is affected by E-cadherin, we tested two additional E-cadherin constructs. The E-cadherin cytoplasm soluble fragment CTF2 (Fig 3A) is a proteolytic cleavage product that naturally occurs during oncogenesis and apoptosis (29). Overexpression of CTF2 results in co-localization of PKD1 and CTF2 in cytoplasm and nucleus (compare Fig 3B and 3D). The IL2R-EcadC (Fig 3A) is a chimera that fuses the extracellular and transmembrane domains of the interleukin-2 receptor α subunit to the cytoplasm domain of E-cadherin (18). Co-expression of the IL2R-EcadC and PKD1 induces co-localization of the two proteins (Fig 3E). We also made a construct that expresses nuclear localized PKD1 by inserting SV40 nuclear localization signal (NLS) into the N-terminus of PKD1 (Fig 2A). This NLS-PKD1 localizes in nuclei in resting cells (Fig 3E). When co-expressed with E-cadherin CTF2 or IL2R-EcadC, the NLS-PKD1 is partially co-localized with the E-cadherin variants in cytoplasm or paranuclear

membranes (compare Fig 3E with 3F and 3G). Taking together, these data strongly suggest that E-cadherin binds to PKD1 and alters PKD1 subcellular localization.

The subcellular localization of activated PKD1 will restrict its biological functions by availability of substrates. We examined PKD1 localization in response to Bryostatin 1 stimulation in the presence or absence of E-cadherin. First, no matter where the initial locations of PKD1 in resting cells, PKD1 become relocated upon stimulation. Figures 2D, 2E and 3E represent PKD1 relocation from cytoplasm, plasma membrane and nuclear, respectively. Second, in the presence of E-cadherin or its variants CTF2 or IL2R-EcadC, activated PKD1 relocation always associates with E-cadherin or its variants (Fig 3). The wild type PKD1 co-localizes with full-length E-cadherin, CTF2 and IL2R-EcadC at resting and stimulated cells (Fig 3C-3E). The prominent example is NLS-PKD1, which is partially associated with CTF2 and IL2R-EcadC in resting cells, but it becomes overwhelmingly co-localized with the two E-cadherin variants after stimulation (Fig 3F and 3G). Although the biological significance of this finding is unknown, it is possible that DAG-induced PKD1 activation would cause PKD1 association with can-related CTF2 fragment and alters PKD1 substrate accessibility.

E-cadherin binding changes the kinetics of PKD1 activation and substrate phosphorylation profiles. We examined the time course of PKD1 activation in C4-2/GFP and C4-2/E-cad cells by immunoblotting pS916. When treated by Bryostatin 1 in C4-2 and C4-2/E-cad cells, the activation course of PKD1 shows dramatically different in the presence and absence of E-cadherin. PKD1 in the control C4-2/GFP cells was slowly activated and it took 2 hours to reach maximum activation (lanes 1-10, Fig 4A). In contrast, PKD1 in the C4-2/E-cad cells responded quickly and reached peak activation in about 5-10 minutes (lanes 11-20, Fig 4A). Like PKC isoforms, prolonged stimulation (> 4 hours) results in PKD1 protein degradation (31). These data suggest that PKD1, in the absence of E-cadherin, has a slow response to stimulation and maintains maximum activation for a short time period. In contrast, E-cadherin binding confers PKD1 a rapid activation and sustainable maximal activity (Fig 4B).

Next, we ask if the binding of active PKD1 to E-cadherin on plasma membrane will change its substrate phosphorylation profile due to subcellular compartmentation. The antibody pMOTIF recognizes preferred PKD1 phosphorylation motif LxRxxpS/T (where p represents phosphorylated residues) (22). The time courses for PKD1 substrate phosphorylation of C4-2/GFP and C4-2/E-cad cells recognized by pMOTIF antibody are quite different (Fig 4C). First, the high molecular weight substrates (100-200 kDa) in C4-2/GFP cells have no phosphorylation at resting and become gradually phosphorylated over time to reach peak phosphorylation in about1-2 hours. The phosphorylation is diminished after 2 hours. Meanwhile, the same protein bands in C4-2/E-cad cells show basal phosphorylation at resting, and maximal phosphorylation can last for at least 4 hours. The difference in kinetics can also be seen at a protein band around 35 kDa (arrowhead in Fig 4C). The protein is maximally phosphorylated between 30 minutes to 4 hours and is one of the major bands in the C4-2/GFP cells, but it is only weakly phosphorylated in C4-2/E-cad cells. Second, the PKD1 phosphorylates unique substrates in C4-2/GFP cells but not in C4-2/E-cad cells (indicated by * in Fig 4C). Finally, two bands show strong phosphorylation in resting C4-2/E-cad cells (arrows in Fig 4C). Activation of PKD1 inhibits the phosphorylation of the two bands as short as 5 minutes. Although the exact mechanism is unknown, one explanation is that the PKD1 basal activity is responsible for phosphorylation in the resting cell, the translocation of PKD1 upon stimulation disassociates PKD1 from its substrates and therefore restricts substrate availability.

We also tracked PKD1 activation and subcellular localization in a time curse in C4-2 and C4-2/PKD1 cells. Staining for total PKD1 in E-cadherin negative C4-2 cells shows that PKD1 is predominantly in cytoplasm in resting cells. The distribution of PKD1 does not change much until stimulation lasts over 1 hour and PKD1 accumulates around paranuclear and a small portion attaches to plasma membrane (white arrow in Fig 5A). The active PKD1 (stained for pS916) is not found in resting C4-2 cells, but it becomes detectable 5 minutes after stimulation and it gradually accumulates on plasma membrane and presumably in trans Golgi Network (30) over the time curse (Fig 5A). In general, the staining patterns of total PKD1 and active PKD1 are partially overlapped but remain different, suggesting not all PKD1 molecules in C4-2 cells are phosphorylated at S916. The C4-2/PKD1 cells stably express wild type PKD1 and induces high level expression of E-cadherin [ref (16) and Fig 5B]. The total PKD1 protein in resting C4-2/PKD1 cells distributes in both cytoplasm and plasma membrane where it co-localizes with E-cadherin (white arrows in Fig 5B). With longer stimulation, more PKD1 moves to plasma membrane. For unknown reasons, the active PKD1 is ready to detect in resting C4-2/PKD1 cells (Fig 5B). The active PKD1 accumulates to plasma and organelle membrane over the time. It is obvious that the staining pattern of active PKD1, in the presence of E-cadherin, is more similar to that of the total PKD1, suggesting there are more S916 phosphorylated PKD1 molecules in C4-2/PKD1 cells than those in C4-2 cells.

DISCUSSION

The E-cadherin/β-catenin complex is a pivotal epithelial adhesion molecule and regulatory mechanism of Wnt signaling. PKD1 has been reported to associate with E-cadherin (14) and β-catenin (23). In this report, we evaluate the impact of E-cadherin on PKD1 activation. Having shown that the membrane anchored PKD1 can be phosphorylated without stimulation (Fig 1B), we hypothesize that the membrane-anchored PKD1 versus cytosolic PKD1 have different sequential events during activation upon DAG stimulation. The current PKD1 activation model suggests that DAG plays three roles in PKD1 activation: (1) binding to C1 domain of PKD1 and promotes its membrane translocation; (2) anchoring PKD1 on plasma membrane through DAG hydrophobic tails; (3) activating PKCs as upstream kinases to trans-phosphorylate PKD1. In the case of membrane anchored PKD1, it seems that the activation is independent of DAG and PKCs. This kind of signal cascade has many similar examples in literature. Yeast pheromone response pathway, for example, involves the activation of MAP kinase cascade through Gβγ complex and scaffold protein Ste5. Pheromone exposure promotes membrane translocation of Ste5, along with associated inactive MAP kinases, and binds to membrane Gβγ and thus the MAP kinases get accessed to proximate upstream kinase Ste20. Membrane recruitment of Ste5 by Gβγ complex can be bypassed with artificial targeting Ste5 to the plasma membrane in the absence of pheromone (32).

In this report, we present evidence that E-cadherin is required for PKD1 optimal membrane attachment and activation. We emphasize the role of E-cadherin in regulation of PKD1 activity and localization. Our major findings are: (1) PKD1 has distinct kinetics of activation in the presence or absence of E-cadherin (Fig 4A and 4B). In the presence of E-cadherin, PKD1 activation is rapid and sustained. In contrast, it takes much longer time (2 hours) for PKD1 to reach maximal activity in response to the same stimulation in the absence of E-cadherin. This difference in kinetics may physiologically cause significant difference for PKD1 in response to short life signals in a cell; and (2) E-cadherin affects PKD1 subcellular localization (Fig 3B and 3C). Since PKD1 is a potential inhibitor for epithelial to mesenchymal transition (16,33), the disruption of PKD1 subcellular localization by loss of E-cadherin may contribute to metastasis. In addition, our data shows that the soluble E-cadherin cytoplasm fragment CTF2 can also bind to and alter PKD1 subcellular localization (Fig 3D).

Although the biological significance of this alteration is still to be evaluated, it is tempting to assume that PKD1 may play different functions in the presence of E-cadherin CTF2 during tumorigenesis; and (3) the PKD1 substrate phosphorylation shows dramatically distinct patterns with or without E-cadherin (Fig 4C), suggesting that PKD1 may phosphorylate different substrates and regulate different signal pathways and biological events in context of E-cadherin.

In non-epithelial cells, PKD1 may partner with other proteins to achieve optimal activation. One example is B cell antigen receptor (BCR) engagement, which leads to activates both PKD1 and Bruton's tyrosine kinase (Btk) (34). It has been reported that PKD1 physically associates with Btk (35) and Btk-deficiency diminishes PKD1 activation (34), suggesting Btk is required for PKD1 activation. However, Haxhinasto and Bishop found that a naturally occurred dominant negative form of Btk (*xid*, which contains point mutant R28C) does not abrogate PKD1 activation in B cells (36) and the authors proposed that the role of Btk is not to directly activate PKD1, but rather to cooperate with PKD1-mediated signals as an adaptor protein (36). The PKD1 protein contains multiple regulatory domains which can recruit interacting proteins. For example, the C-termini of PKD1 and PKD2 have a PDZ-binding motif and both isoforms can bind to membrane localized PDZ domain-containing protein NHERF-1 (37). Importantly, agonist-evoked activation of PKD in the presence of the NHERF-1 scaffold is rapid and sustained compared with cytosolic PKD (37). In HEK293 cells, the C1b domain of PKD1 directly binds to G-protein Galphaq subunit, and the binding contributes to long-lasting activation of PKD1 on plasma membrane (38). Therefore, our results provide an alternative explanation for the diverse functions of PKD1, i.e. protein-protein interactions are a mechanism for PKD1 regulation, poising PKD1 near upstream kinases and lipid regulators and near specific substrates.

Taken together, our data suggest that E-cadherin provides PKD1 a membrane anchorage and facilitates PKD1 activation in epithelial cells through the interaction of PKD1 with E-cadherin cytoplasm domain. Since PKD1 can suppress the function of transcription factor Snail, a known repressor of E-cadherin expression and up-regulate E-cadherin expression (16), the two proteins form a positive feedback loop that involves transcriptional as well as kinase regulation, favoring maintenance of epithelial cell identity. Loss of E-cadherin expression or cancer-related proteolysis of E-cadherin that generates soluble CTF2 can alter PKD1 localization and function. Our results demonstrate that spatial and temporal organization of the PKD1 signal transduction is essential in determining the speed and precision by which signaling events occur.

ACKNOWLDEGEMENTS: This work was partially supported by Department of Defense grant award W81XWH-11-1-0282.

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Figure Legends

Figure 1. E-cadherin interacts with PKD1. (A) Yeast 2-hybrid assay for direct interaction of PKD1 and E-cadherin. Individual PKD1 domain indicated in the figure and E-cadherin intracellular domain were used for testing. Plate on right side is grown on non-selective medium and the plate on the left is grown on selective medium. Schematic of PKD1 structure is shown at lower panel. (B) Co-precipitation of PKD1 and E-cadherin. The C4-2/PKD1 cells stably overexpress GFP tagged wild type PKD1 and induce E-cadherin expression (16). C4-2/PKD1 cell lysate is used for reciprocal IP. (C) Knockdown E-cadherin reduces PKD1 activation. ShRNA targeting E-cadherin or control (siLuc) were transit transfected into E-cadherin positive cell line LNCaP. Cells were cultured for another 48 hours and treated with 20 nM of Bryostatin 1 for 30 minutes. Cell lysates were used for Western blotting analysis for indicated antibodies. All results are from a single experiment, with similar results obtained in another experiment. (D) Immunofluorescent staining of PKD1 in LNCaP cells. LNCaP cells were co-transfected by GFP tagged wild type PKD1 (green) and shRNA vector targeting either luciferase (shLuc, control) or E-cadherin (shE-cad) for 48 hours before stimulating by Bryostatin (20 nM) for 30 minutes. Representative images are shown. Nucleus is in red.

Figure 2. Artificial targeting to plasma membrane activates PKD1. (A). Schematic of PKD1 constructs used in this study. Wild-type PKD1: full length human PKD1 tagged with GFP. Mem-PKD1: inserting the first 20 amino acid residues of neuromodulin that contains a palmitoylation signal and leads the fusion proteins to membranes upstream of GFP-PKD1. P281G-PKD1: PKD1 containing a point mutation on proline-281 to glycine. P281G/mem-PKD1: mem-PKD1 containing a point mutation of P281G. NLS-PKD1: PKD1 with a nuclear localization signal. (B) Targeting of PKD1 to the membrane activates PKD1 without stimulation.

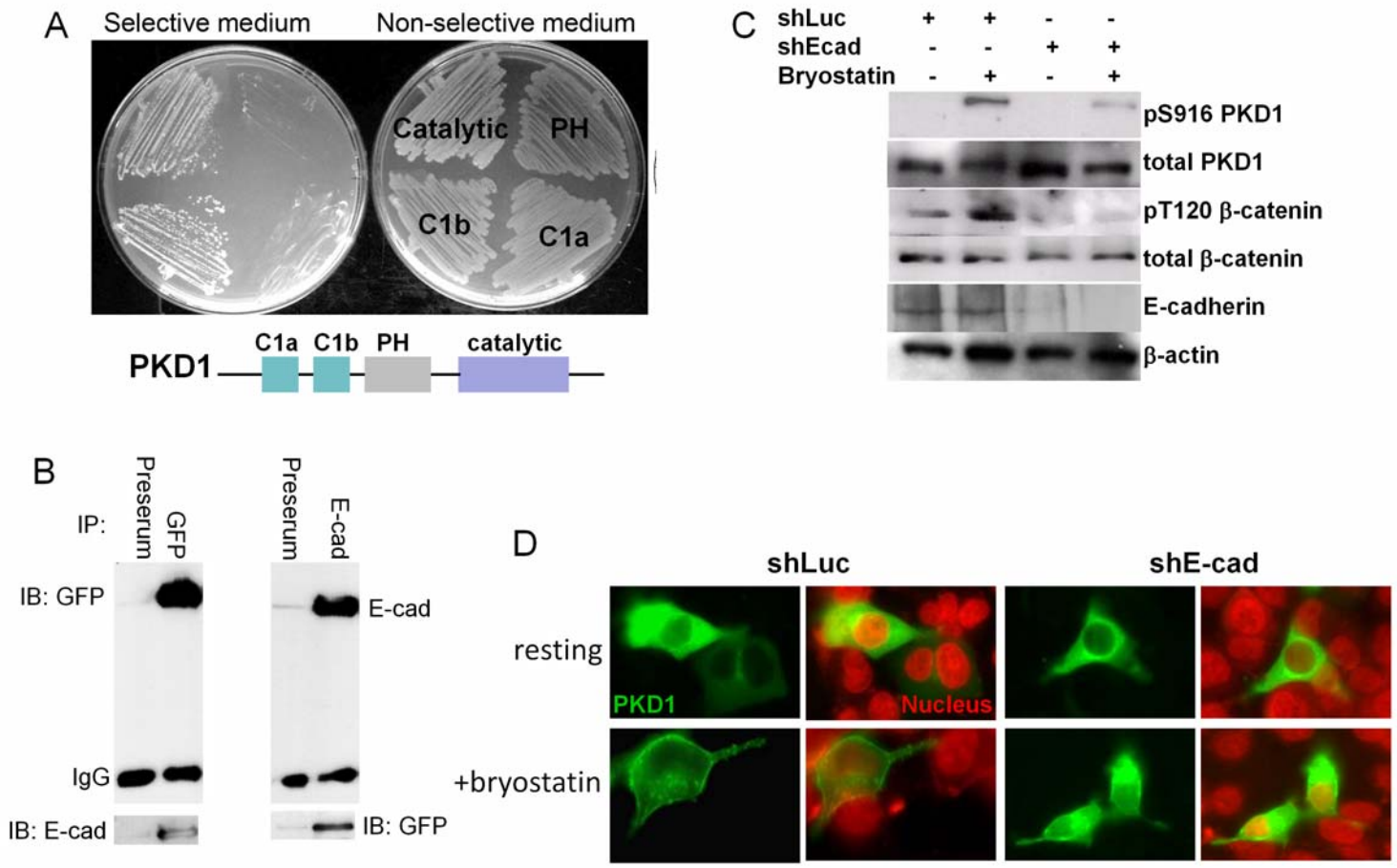
Indicated constructs were transiently transfected into C4-2 cells for 48 hours. Bryostatin 1 was added at final concentration of 20 nM for 30 minutes. (Top panel) Immunoblotting with PKD1 pS916 antibody. Endo- and exo- represent endogenous and GFP-tagged exogenous PKD1, respectively. The endogenous PKD1 can be used as a wild type PKD1 control. (Lower panel) immunoblotting for total PKD1. Data comes from a single experiment, with similar results obtained in another experiment. (C) Activation of membrane attached PKD1 is PKC-independent. C4-2 cells were transfected as in (B). The transfected cells were pre-treated with general PKC inhibitor GF1 for 2 hours (final concentration 2.5 μM) before Bryostatin was added. (Top panel) Immunoblotting with PKD1 pS916 antibody. (Lower panel) immunoblotting for total PKD1. Data comes from a single experiment. (D) GFP-tagged wild type and (E), mem-PKD1 were transit transfected into SW480 cells. Representative immunofluorescent images are taken for resting cells (top panel) and bryostatin (20 nM) treated cells (lower panel). Nucleus is in red.

Figure 3. PKD1 is associated with E-cadherin in resting and Bryostatin treated cells. (A) Schematic of E-cadherin constructs used in this study. (top) wild type E-cadherin; (middle) E-cadherin soluble cytoplasm fragment CTF2; and (bottom) the CTF2 fused to the transmembrane domain of Interleukin 2 receptor. (B-E) GFP tagged wild type PKD1(WT-PKD1) was either transit transfected alone (B), or co-transfected with full-length E-cadherin (C), or CTF2 (D), or IL2R-EcadC (E) into SW480 cells and cultured for another 48 hours. (F-H) GFP-tagged nuclear localized PKD1 (NLS-PKD1) was either transit transfected alone (E), or with E-cadherin CTF2 (G), or with IL2R-EcadC (H). Bryostatin (20 nM final concentration) was added for 30 minutes before fixation and staining. Representative immunofluorescent images are shown. Nucleus is in blue.

Figure 4. E-cadherin confers rapid and sustained activation for PKD1 and unique substrate phosphorylation profiles. C4-2 cells that stably express GFP or full-length E-cadherin were treated with 20 nM Bryostatin 1 for indicated times. Cell lysates were used for immunoblotting for total and active (pS916) PKD1 (A), PKD1 phosphorylated substrate (C). All results are from a single experiment, with similar results obtained in another experiment. (B) PKD1 activation was quantified by Image J software. The chart was based single experiment.

Figure 5. Time course of PKD1 activation in C4-2 and C4-2/PKD1 cells. C4-2 (A) and C4-2/PKD1 (B) cells were cultured in glass slides for 24 hours before adding Bryostatin 1 (20 nM) for indicated times. Total PKD1 (left panels) and active PKD1 (right panels) were stained. Representative immunofluorescent images are shown. Nucleus is in blue.

Fig 1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Selective medium
Non-selective medium
Catalytic
PH
C1b
C1a
PKD1
C1a C1b PH catalytic
B
IP:
Preserum
GFP
Preserum
E-cad
IB: GFP
E-cad
IgG
IB: E-cad
IB: GFP
C
shLuc + + - -
shEcad - - + +
Bryostatin - + - +
pS916 PKD1
total PKD1
pT120 β-catenin
total β-catenin
E-cadherin
β-actin
D
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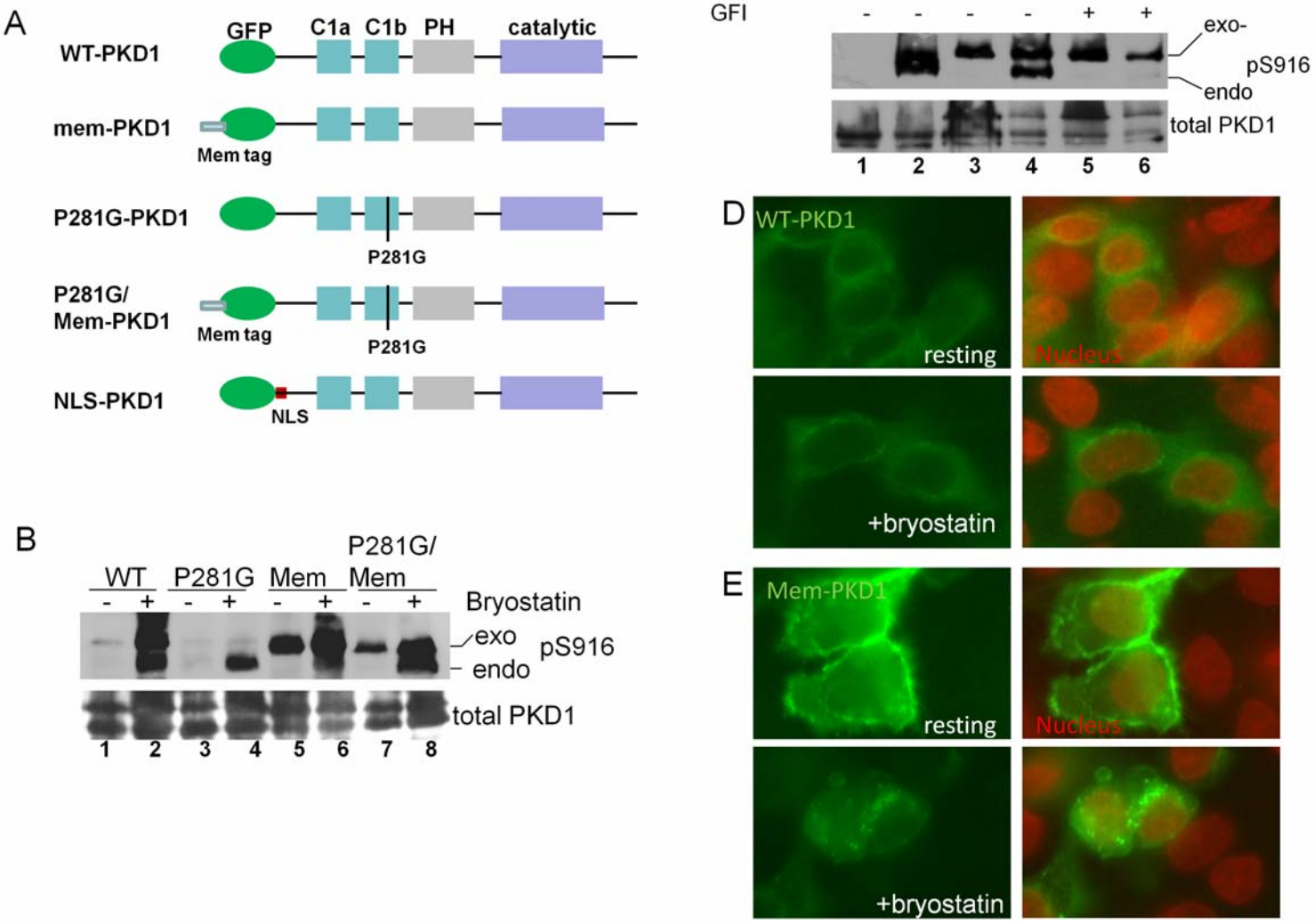
shE-cad
resting
+bryostatin
PKD1
Nucleus

Fig 2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GFP
C1a
C1b
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catalytic
WT-PKD1
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Mem tag
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P281G
P281G/
Mem-PKD1
Mem tag
P281G
NLS-PKD1
NLS
B
WT
P281G
Mem
P281G/
Mem
- + - + - + - +
Bryostatin
exo
endo
pS916
total PKD1
1 2 3 4 5 6 7 8
C
Mem-PKD1 - - + + + +
Bryostatin - + - + - +
GFI - - - - + +
exo-
pS916
endo
total PKD1
1 2 3 4 5 6
D
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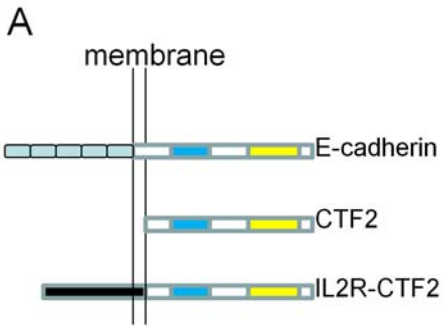
resting
Nucleus
+bryostatin
E
Mem-PKD1
resting
Nucleus
+bryostatin

Fig 3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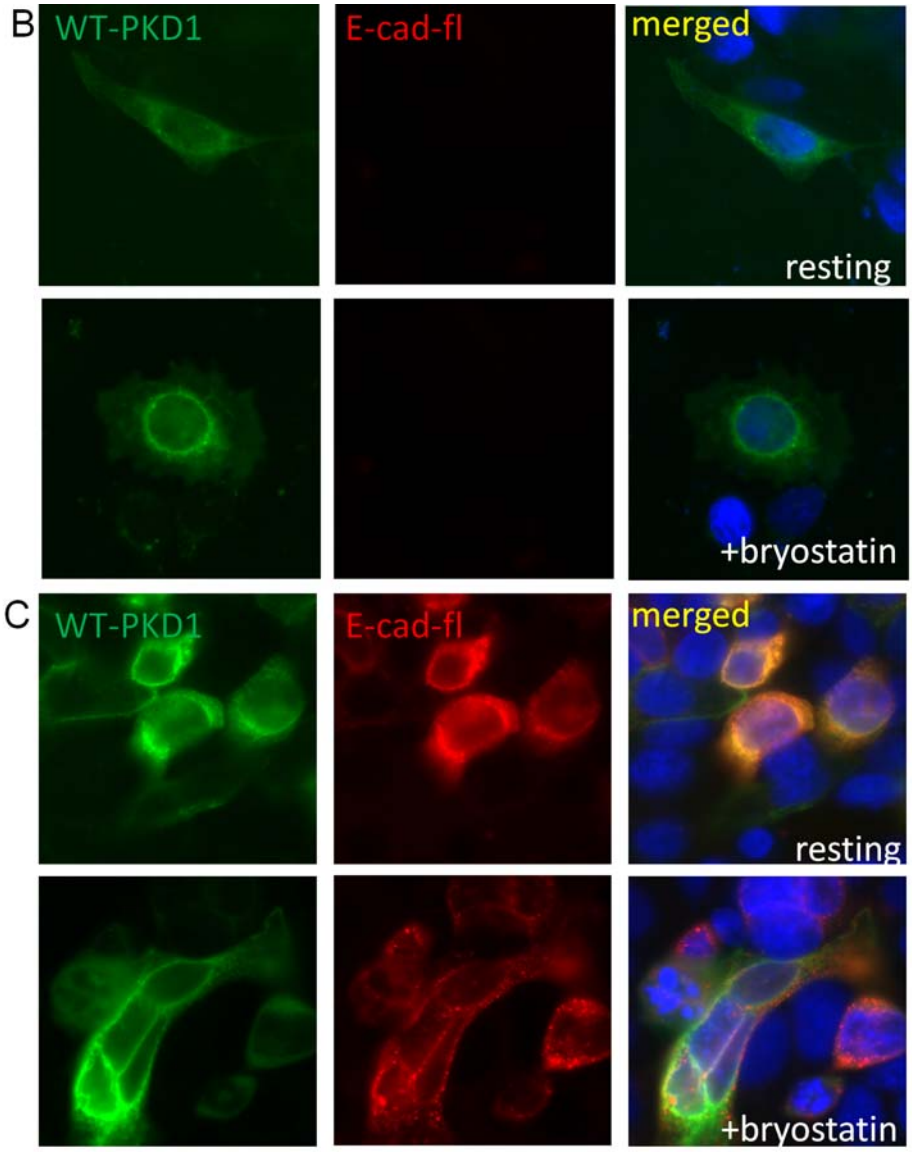
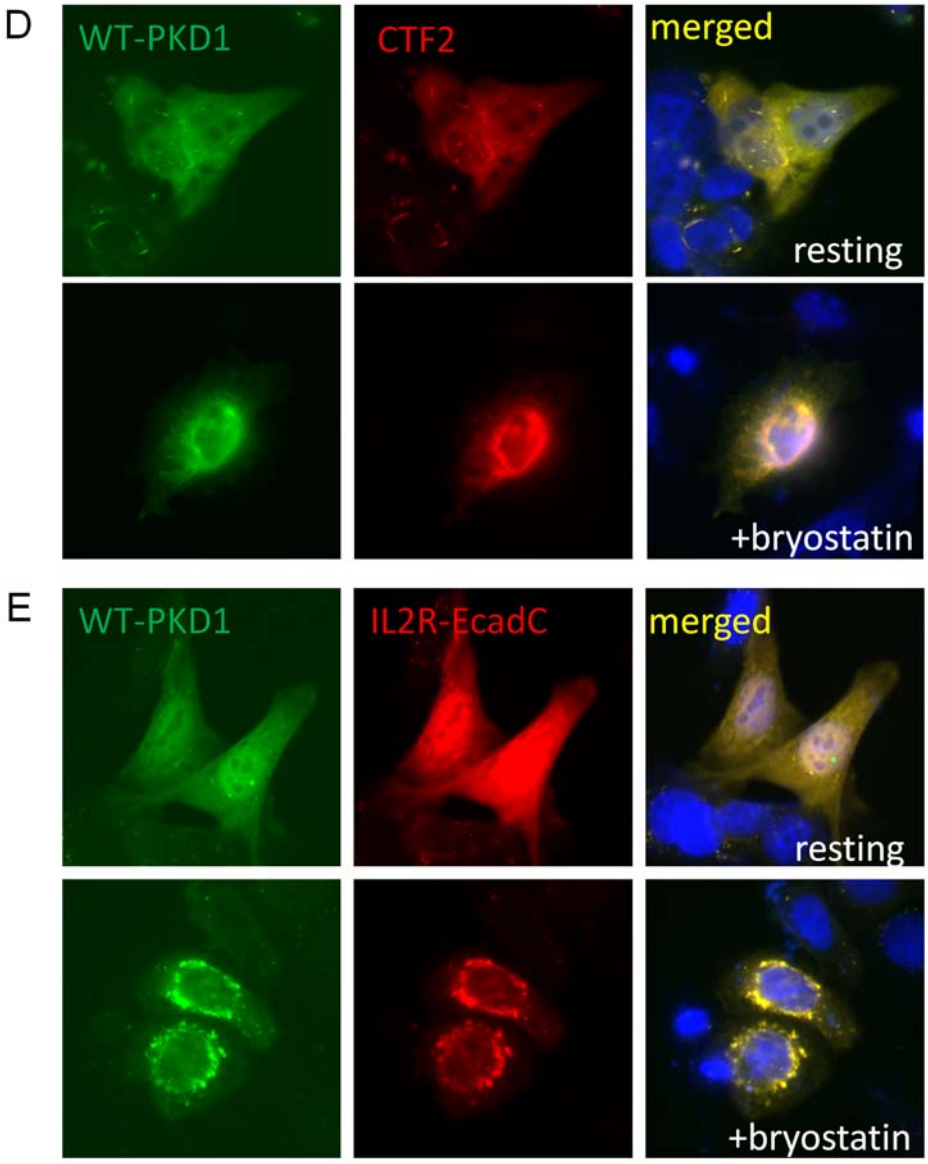
E-cadherin
CTF2
IL2R-CTF2


B
WT-PKD1
E-cad-fl
merged
resting
+bryostatin
C
WT-PKD1
E-cad-fl
merged
resting
+bryostatin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WT-PKD1
CTF2
merged
resting
+bryostatin
E
WT-PKD1
IL2R-EcadC
merged
resting
+bryostatin

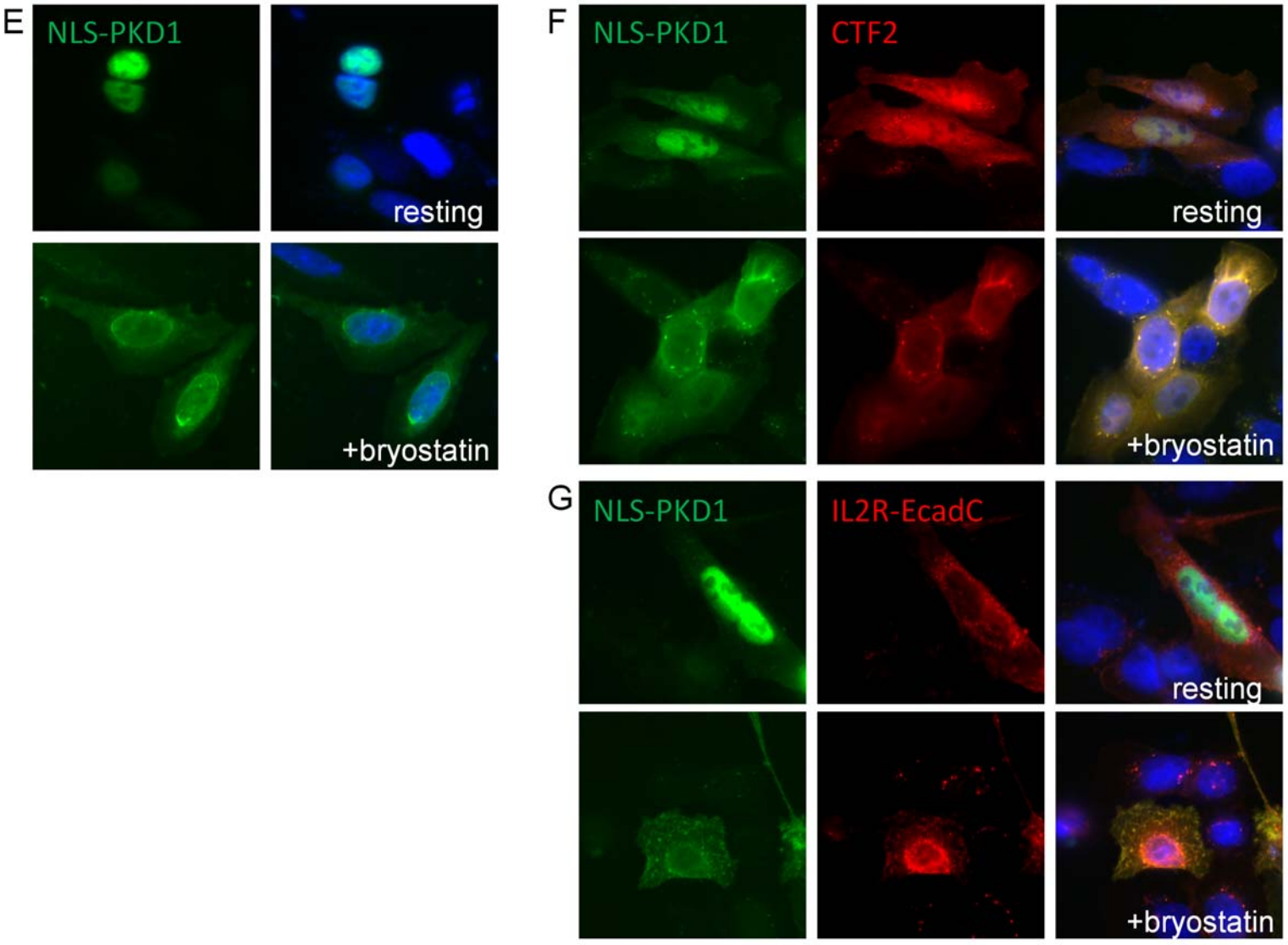
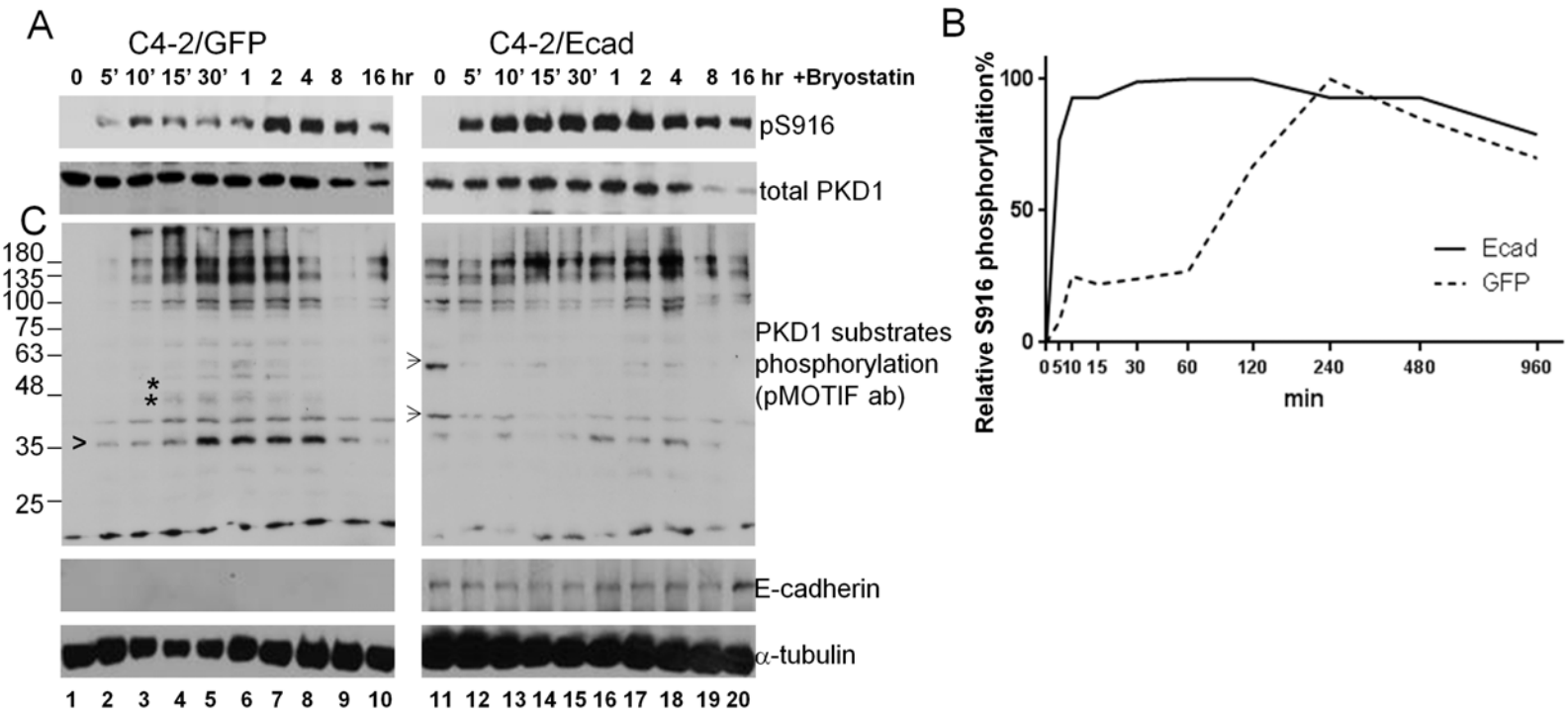

E
NLS-PKD1
resting
+bryostatin
F
NLS-PKD1
CTF2
resting
+bryostatin
G
NLS-PKD1
IL2R-EcadC
resting
+bryostatin

Fig 4


A
C4-2/GFP
C4-2/Ecad
0 5' 10' 15' 30' 1 2 4 8 16 hr
0 5' 10' 15' 30' 1 2 4 8 16 hr +Bryostatin
pS916
total PKD1
C
180
135
100
75
63
48
35
25
PKD1 substrates phosphorylation (pMOTIF ab)
E-cadherin
α-tubulin
1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20
B
Relative S916 phosphorylaition%
100
50
0
Ecad
GFP
0 5 10 15 30 60 120 240 480 960
min

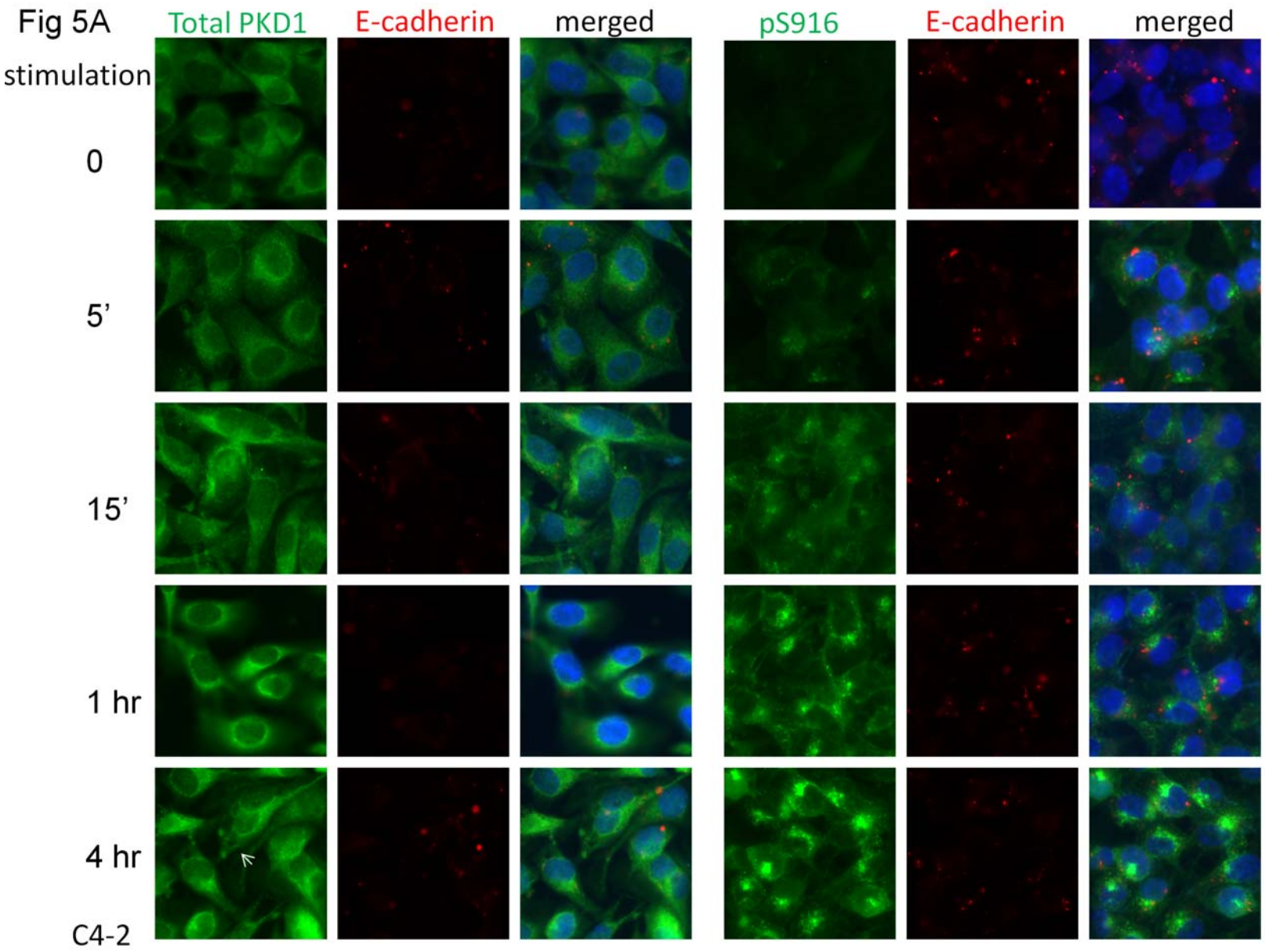


Fig 5A
Total PKD1
E-cadherin
merged
pS916
E-cadherin
merged
stimulation
0
5'
15'
1 hr
4 hr
C4-2

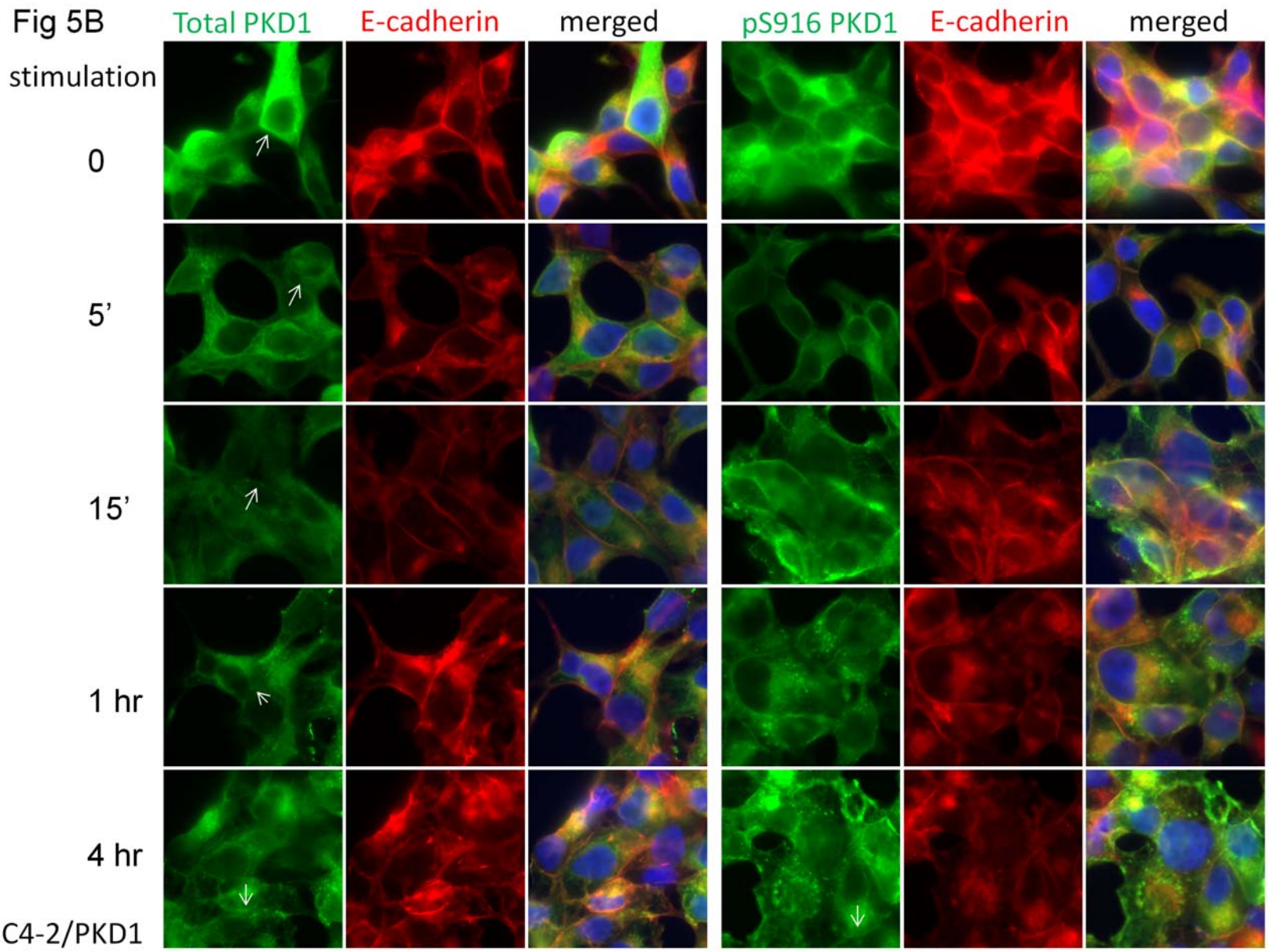


Fig 5B
Total PKD1
E-cadherin
merged
pS916 PKD1
E-cadherin
merged
stimulation
0
5'
15'
1 hr
4 hr
C4-2/PKD1